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FROM STATENS SERUMINSTITUT, AMAGER BOULEVARD 80 COPENHAGEN S DENMARK STREPTOCOCCAL DEPARTMENT, (CHIEF KNUD SKADHAUGE M D)

INFECTION AND MALIGNANT TUMOURS

5 *Inhibition of Brown-Pearce Carcinoma by Cell-Free Extract of Haemolytic Streptococci Prepared by Grinding the Bacteria with Alumina*

By

EBBE AHRENSBURG CHRISTENSEN

Received 21 1 63

In previous publications (3, 4, 5, 6) it was reported that a bacteria-free lysate of haemolytic streptococci, obtained by lysing the culture with streptococcal phage, could inhibit the growth of Brown-Pearce carcinoma in young rabbits These publications included a brief review of earlier investigations on the antagonism between infection and malignant tumours

The present study deals with the inhibitory effect on Brown-Pearce carcinoma in young rabbits of a cell-free extract of haemolytic streptococci which was prepared by grinding the bacteria with powdered alumina by the method reported by *McIlwain* in 1948 The same technique, but followed by precipitation with acetone, was used by *Koshimura & Shoin* in 1960 to prepare a cell free extract of haemolytic streptococci This extract could prevent the take of Ehrlich carcinoma in mice, if the carcinoma cells were incubated with the extract for 90 minutes at 37° C before being transplanted

MATERIAL AND METHODS

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After transplantation each litter was divided into groups so that the control animals (treated with placebo or untreated) were from the same litter as those treated with extracts of haemolytic streptococci

Isotonic phosphate buffer saline pH 7 38 was used as placeb...

Extracts were ...

(1 ... 12 no 3465 cer ... h (11) were ... 30 minutes in a cooled porcelain mortar w... ...c bacterial mass was gro... (T nerde no 3 prepared for the purp... & C... Dusseldorf) The mixture was th...

This study has been aided by grants from *P Carl Petersen s Fund*

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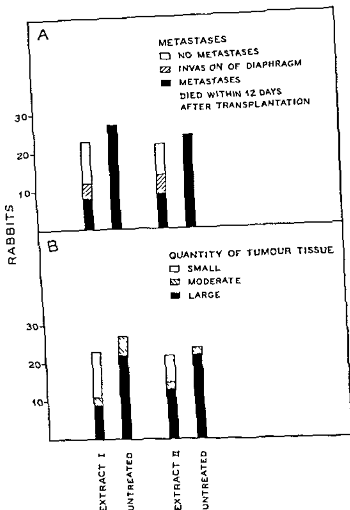


Fig 1 A and B

Metastasization and quantity of tumour tissue after treatment with extract I (from streptococci grown in human serum broth) and extract II (from streptococci grown in trypsin broth) respectively

Both extracts were found to inhibit growth of the tumour and its ability to metastasize in a number of the animals treated (Fig 1 A & B) No definite difference was found in the tumour inhibiting effect of the two different extracts, and neither in toxicity nor in yield per liter culture did the one extract show any advantage over the other

As the production of extract II does not involve the use of human serum all subsequent extracts were prepared as extract II, *i e* from culture in trypsin broth

Two out of 16 extracts prepared in this way had no demonstrable tumour inhibiting effect One of these two extracts was found to be

isotonic phosphate buffer saline, pH 7 38 for 24 hours each time The extract was sterilized by filtration through a washed Berkefeld filter The final product was clear, faintly yellowish-green, frothy, pH 7 6–7 8 All procedures were carried out at temperatures between 0° C and 8° C, and the finished preparations were stored at —16° C until use

All injections were administered by the intraperitoneal route The first injection was made on the second day after transplantation at the earliest When more than one injection was given the schedule was as follows three injections given as one injection weekly for three weeks, six injections, given as two injections weekly for three weeks and nine injections, given as three injections weekly for three weeks

The general condition of the animals was judged on their increase in weight and on their appearance All animals were weighed on the day of transplantation, three days later, subsequently once a week and finally on the day of death

The rapid growth of the tumour in the peritoneum and the occurrence of metastases as early as the 11th day after transplantation made it possible to terminate the experiments between the 24th and 30th day after transplantation

Autopsy of all the rabbits was carried out with the same technique and by the same examiner On the basis of the autopsy findings, the animals were divided into three groups according to the occurrence of metastases (A) and into three groups according to the quantity of tumour tissue (B) as described earlier (6)

A 1 Tumour growth restricted to peritoneum
  2 Tumour growth in peritoneum and invasion of diaphragm to pleura
  3 Metastases at one or more sites
B 1 No or little tumour tissue
  2 Moderate quantity of tumour tissue
  3 Large quantity of tumour tissue

RESULTS

Two extracts were prepared from haemolytic streptococci grown in human serum broth (extract I) and 16 extracts from growth in trypsin broth (extract II)

In preparing the extracts the proportion was varied between the amount of bacteria used and the amount of extracting solution, so as to give 1 ml of extract from between 0 03 and 0 10 g of bacteria (wet weight) The experimental results showed no differences which might be ascribed to these variations in the amount of extracting solution In the present experiments, the tumour-inhibiting effect depended only on the amount of bacteria used for the preparation with which the animals were treated

In the *first series of experiments*, two extracts from streptococci grown in human serum broth (extract I) were compared with two extracts from cultures in trypsin broth (extract II) Twenty-four animals were treated with various doses of extract I and 24 with corresponding doses of extract II In all cases three injections were given weekly for three weeks Untreated animals were used as controls

Of a total of 99 animals, three died before the 12th day after transplantation These animals were excluded from the material, since they were not comparable with the other animals with respect to quantity of tumour tissue and presence of metastases One of these three animals was being treated with extract I and the other two had been given very large single doses of extract II

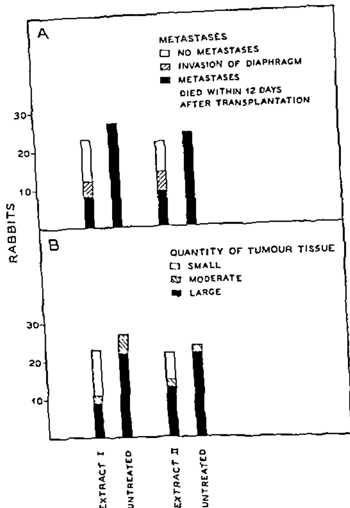


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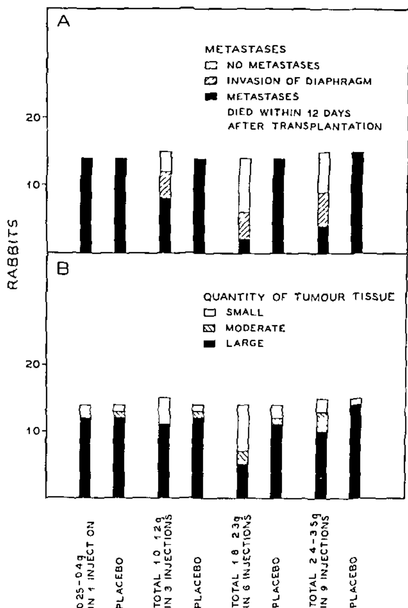


Fig 2 A and B

Metastasization and quantity of tumour tissue after treatment with a varying number of injections with the same amount of extract per injection Dose indicated in g of bacteria (wet weight)

contaminated with non-motile Gram-negative rods which could multiply at 4° C The observation has previously been reported (4) that a phage-lysed culture of haemolytic streptococci can lose its tumour-inhibiting effect as a result of bacterial contamination No explanation could be found for the lack of effect in the case of the other extract The remaining 14 preparations all appeared to have the same tumour inhibiting *effect and toxicity, i e any possible differences between the* preparations were too small to appear in the effect on the animals

In the *second series of experiments,* the animals were treated with

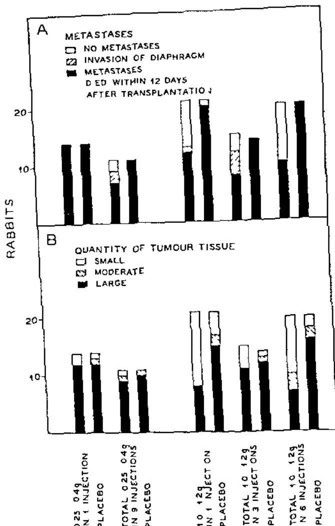


Fig 3 A and B

Metastasization and quantity of tumour tissue after treatment with the same total dose given either as one injection or distributed over a number of injections Dose indicated in g of bacteria (wet weight)

one, three six and nine injections respectively, using the same amount of extract per injection The first injection was given on the 2nd day after transplantation

One injection had no effect, but three, six and nine injections inhibited growth of tumour and metastasization in a number of the animals The most pronounced inhibition was observed in those animals which received a total of six injections and not in those which received the maximum total dose, *i e* nine injections (Fig 2 A & B) However, in view of the limited material, this difference between the effect of six

and nine injections, respectively, is uncertain On the other hand, since it is in agreement with the experience from a previous investigation using phage-lysed cultures of streptococci (4), it is reasonable to suppose that the difference is a real one

In the *third series of experiments*, the effect of a single injection was compared with the effect of the same total amount of extract distributed over three, six or nine injections

In the second series of experiments it had, as mentioned, been found that a single injection on the 2nd day after transplantation was without effect on the tumour It now appeared that the same amount of extract spread over nine injections, had only a doubtful tumour-inhibiting effect (Fig 3 A & B) It seemed likely, therefore, that the size of the total dose was more decisive for the effect than the number of injections

Previous experiments have shown that even as late as the 8th day after transplantation a series of injections could be commenced which resulted in definite tumour-inhibition (4) By the 8th day after transplantation the young rabbits had become more robust and, if a single injection were given at that stage, the dose could be tripled

On the basis of these observations 21 rabbits were given a single large dose on the 8th day Tumour inhibition was achieved in a considerable proportion of the animals which had been treated with a dose of the extract corresponding to 1 2 g of bacteria (wet weight) The results were the same whether this dose was given as a single injection or distributed over three or six injections (Fig 3 A & B)

TABLE 1

Treatment	Number of rabbits			
	at commencement of experiment	dead before 12th day after transplantation	with adequate observation period	without vital tumour tissue at autopsy
Extract from 3 0–6 5 g bact *	17	3	14	5
Placebo	15	1	14	0
Extract from 2 0–3 0 g bact	34	0	34	8
Placebo	15	0	15	0
Extract from 1 0 2 0 g bact	78	2	76	23
Placebo	56	0	56	0
Extract from 0 5 1 0 g bact	33	0	33	6
Placebo	23	0	23	1
Extract from 0 25 0 5 g bact	15	1	14	1
Extract from 0 1 g bact	13	0	13	0
Placebo	10	0	10	0
No treatment	67	1	66	0

* Wet weight

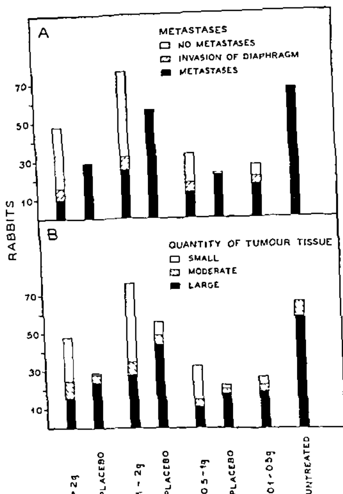


Fig 4 A and B

Metastasization and quantity of tumour tissue after treatment with various doses
Dose indicated in g of bacteria (wet weight)

An analysis was made of all trials with the extracts to elucidate the relationship between the size of the dose and the inhibitory effect on tumour growth and metastasization All 16 active preparations (two extract I and 14 extract II) had been tried in various doses to compare the effect of the different extracts The analysis included all animals which had been treated with six or nine injections of one of the extracts together with the corresponding control animals, no matter whether the animals were also included in other of the experimental series A total of 376 animals was involved 190 treated with extract, 119 treated with placebo and 67 untreated animals

A total of 8 of these 376 animals had died before the 12th day after

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On the basis of these observations 21 rabbits were given a single large dose on the 8th day. Tumour-inhibition was achieved in a considerable proportion of the animals which had been treated with a dose of the extract corresponding to 1.2 g of bacteria (wet weight). The results were the same whether this dose was given as a single injection or distributed over three or six injections (Fig. 3 A & B).

TABLE 1

Treatment	Number of rabbits			
	at commencement of experiment	dead before 12th day after transplantation	with adequate observation period	without vital tumour tissue at autopsy
Extract from 3.0–6.5 g bact.*	17	3	14	5
Placebo	15	1	14	0
Extract from 2.0–3.0 g bact.	34	0	34	8
Placebo	15	0	15	0
Extract from 1.0–2.0 g bact.	78	2	76	23
Placebo	56	0	56	0
Extract from 0.5–1.0 g bact.	33	0	33	6
Placebo	23	0	23	1
Extract from 0.25–0.5 g bact.	15	1	14	1
Extract from 0.1 g bact.	13	0	13	0
Placebo	10	0	10	0
No treatment	67	1	66	0

* Wet weight

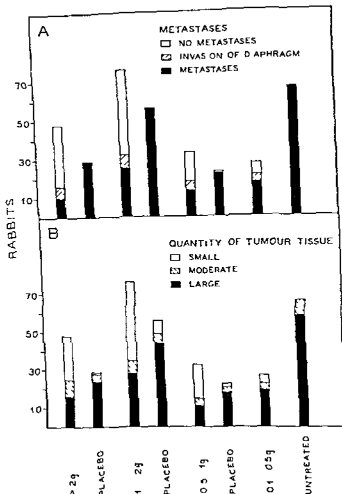


Fig 4 A and B

Metastasization and quantity of tumour tissue after treatment with various doses Dose indicated in g of bacteria (wet weight)

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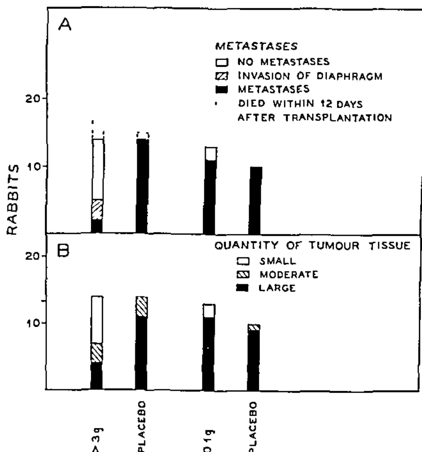


Fig 5 A and B

Metastasization and quantity of tumour tissue after treatment with very large and very small doses Dose indicated in g of bacteria (wet weight)

transplantation and was therefore excluded from the material Six of these rabbits were being treated with extract, one was being treated with placebo and one animal was untreated (Table 1)

Doses which were the equivalent of from 0 5 to 3 0 g of bacteria (wet weight) had resulted in pronounced inhibition of the tumour in 50 to 70 per cent of the animals (Table 2 and Fig 4 A & B), and no impairment in the general health condition of the animals was observed Just as in the experiments with phage-lysed streptococci (6, 4), absence of metastases proved to be the most sensitive criterion for the inhibitory effect on the Brown-Pearce carcinoma

In 17 animals, the intention had been to administer a total dose corresponding to more than 3 g of bacteria (wet weight) (between 3 0 and 6 5 g) Three of these 17 animals had died before the 12th day after transplantation, and a further four died before the termination of the period of the experiment These very large doses were, as the mortality shows, more than the animals could *tolerate* Tumour-inhibition was *no more* pronounced than it was with moderate doses (Table 2 and Fig 5 A & B)

A total of 13 animals was treated with doses corresponding to ap-

TABLE 2

The tumour-inhibiting effect of various doses of extract from haemolytic streptococci

Treatment	Number of rabbits							
	without metastases at autopsy		with invasion of diaphragm at autopsy		with metastases at autopsy		with no or little tumour tissue at autopsy	
	fraction of total	per cent	fraction of total	per cent	fraction of total	per cent	fraction of total	per cent
Extract from 3 0 6 5 g bact *	9/14	(64)	3/14	(22)	2/14	(14)	7/14	(50)
Extract from 2 0–3 0 g bact	23/34	68	3/34	9	8/34	23	16/34	47
Extract from 1 0–2 0 g bact	44/76	58	7/76	9	25/76	33	41/76	54
Extract from 0 5 1 0 g bact	15/33	45	5/33	15	13/33	40	18/33	55
Extract from 0 25–0 5 g bact	4/14	(29)	4/14	(29)	6/14	(42)	2/14	(14)
Extract from 0 1 g bact	2/13	(15)	–	–	11/13	(85)	2/13	(15)

* Wet weight

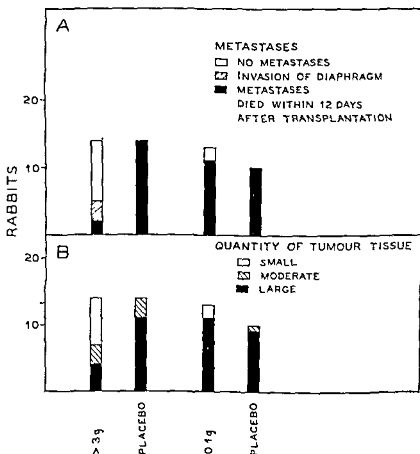


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A total of 13 animals was treated with doses corresponding to ap-

As mentioned, tumour-inhibition in the second experimental series was less pronounced after nine injections (i e the largest total dose) than after six injections This phenomenon which is presumably real as the same tendency was observed on more than one occasion, may possibly be interpreted either as an inhibition or blocking of a defence mechanism against the tumour on the part of the rabbit, or as a stimulation of the adrenal cortex in the host animal Both possibilities would lead to accelerated growth of the tumour, the latter alternative in particular to an earlier and more frequent metastasization Similar phenomena have been observed in the treatment of other tumour/host systems by various macromolecular substances (1, 2) In the present experiments, this phenomenon and the tumour inhibiting effect need not be dependent on the same factor The extracts contain a number of different substances, and the moderately accelerating effect of the large doses on the tumour might be due to a substance which, in relation to the tumour inhibiting effect, must be regarded as an impurity Supporting this possibility is the fact that the phenomenon was seen with only two of the extracts and did not appear in the analysis of the total material *Placebo treatment with human serum broth* has a similar, weakly pronounced, accelerating effect on the growth of the tumour in comparison with untreated control animals (6), while phosphate buffer saline appears to be quite indifferent as placebo

SUMMARY

Young rabbits with Brown-Pearce carcinoma have been treated with extracts from mechanically broken haemolytic streptococci

Using large doses, a blocking of the metastasizing power and a pronounced inhibition of the tumour growth was observed in 50 70 per cent of the animals treated

No difference was observed in the effect of extracts from streptococci grown in human serum broth and extracts from growth in trypsin broth

The extracts were active both as a single injection and as a series of injections

Very large doses, which caused death in about 1/3 of the animals, resulted in no more pronounced inhibition of the tumour than moderate doses

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proximately 0.1 g of bacteria (wet weight), i.e. approximately 1/10 of the optimum. This dose was too small to permit a definite tumour-inhibition to be observed (Fig. 5 A & B).

No vital tumour tissue was found at autopsy (macroscopic examination) in 42 out of a total of 157 animals treated with extract corresponding to more than 0.5 g of bacteria (wet weight). Otherwise, only two of the remaining 211 animals in the analysis showed no vital tumour tissue at autopsy: one out of a total of 27 animals which had been treated with extract corresponding to less than 0.5 g of bacteria (wet weight), and one out of a total of 118 placebo treated animals (Table 1).

DISCUSSION

The experiments described here have shown that a bacteria free extract prepared from mechanically ground streptococci inhibits the growth of Brown Pearce carcinoma in young rabbits in the same way as phage lysed cultures of streptococci (4, 5, 6). However, the inhibitory effect of the extracts employed in the present study was lower than that obtained with phage-lysed cultures. The number of optimal doses which could be prepared from one liter of bacterial culture represented only between one half and one fifth of the number which was obtained from one liter of phage lysed culture of streptococci.

However, compared to the phage lysed cultures, the extracts had the advantage of being somewhat less toxic and more uniform from preparation to preparation. Furthermore, the extracts could be made more concentrated than the lysates, so that the tumour-inhibiting effect could be achieved with smaller amounts of solution. However, the margin of therapeutic safety was narrow in both cases.

The production of various known extracellular toxins and enzymes in haemolytic streptococci is very dependent on the environmental conditions (9). *The experiments described here have shown that the strain* of streptococci employed produces the tumour inhibiting factor in the absence of human serum. This point had not been examined previously, as all phage-lysed cultures have been grown in human serum broth.

It was found previously in experiments with phage-lysed cultures that a single injection had no effect on the growth of tumour and on metastasization (4). A possible explanation was that the dose with bacterial lysates could not be increased sufficiently without the appearance of toxic effects. The present experiments strongly support this assumption, as a sufficiently large dose of extract resulted in tumour-inhibition both as a single injection and distributed over three or six injections. The material is too small to decide whether the same total dose has the same quantitative effect when given as one injection or distributed over several injections, but the tendency in the experiments indicates that several injections have a more pronounced tumour-inhibiting effect.

As mentioned tumour inhibition in the second experimental series was less pronounced after nine injections (i e the largest total dose) than after six injections This phenomenon which is presumably real as the same tendency was observed on more than one occasion, may possibly be interpreted either as an inhibition or blocking of a defence mechanism against the tumour on the part of the rabbit, or as a stimulation of the adrenal cortex in the host animal Both possibilities would lead to accelerated growth of the tumour, the latter alternative in particular to an earlier and more frequent metastasization Similar phenomena have been observed in the treatment of other tumour/host systems by various macromolecular substances (1, 2) In the present experiments, this phenomenon and the tumour-inhibiting effect need not be dependent on the same factor The extracts contain a number of different substances, and the moderately accelerating effect of the large doses on the tumour might be due to a substance which, in relation to the tumour inhibiting effect, must be regarded as an impurity Supporting this possibility is the fact that the phenomenon was seen with only two of the extracts and did not appear in the analysis of the total material Placebo treatment with human serum broth has a similar, weakly pronounced, accelerating effect on the growth of the tumour in comparison with untreated control animals (6), while phosphate buffer saline appears to be quite indifferent as placebo

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FROM THE UNIVERSITY INSTITUTE OF FORENSIC MEDICINE COPENHAGEN DENMARK (HEAD PROFESSOR H GORMSEN M.D)

FATAL SUBARACHNOID HAEMORRHAGE ORIGINATING IN AN INTRACRANIAL CHORDOMA

By

J SIMONSEN

Received 29 1 63

Among the aetiological factors of subarachnoid haemorrhage (sacculated aneurysms angiomas, atheromatous changes tumours, etc) intracranial tumours make up only a very small group

In an autopsy series of 62 cases of fatal subarachnoid haemorrhage *Wolfe* (1953) found only one in which the haemorrhage had originated in an intracranial tumour, an astrocytoma Of *Walton's* (1953) large series of 312 cases 4 were due to intracranial tumours, a glioma, a meningioma, and in two cases metastases, from a bronchogenic carcinoma and a melanoma respectively In a medico legal autopsy series of 126 cases of sudden death from subarachnoid haemorrhage *Simonsen* (1963) found one in which the haemorrhage had originated from a tumour a chordoma

The literature also contains single case reports of fatal subarachnoid [illegible] ntracranial tumours Glio- [illegible] (*Echols & Rehfeldt* 1950), [illegible] *stensen & Larsen* 1953)

Apart from the case mentioned in a previous review by the present author (1963), no other case of subarachnoid haemorrhage due to a chordoma seems to be on record The case will, therefore, be reported below in some detail Owing to the rare occurrence of chordomas on the whole and the relative rarity of massive haemorrhage in intra cranial tumours the embryology and pathology of chordoma will be recapitulated and a few factors regarding the cause of death in intra cranial tumours in general will be briefly discussed

CASE REPORT

(R I F 126 60) A 38 year old woman who ha[illegible] athel[illegible] [illegible]

[illegible] complained of a severe head [illegible] that she was about to collapse the doctor bent her head down towards her knees Her pulse became fast and thready and Cheyne Stokes respiration supervened Despite artificial respiration and injection of adrenaline (0 5 ml) intra venously and later intracardially death occurred almost instantaneously

Fig 1
Area showing a cartilaginous appearance (haematoxylin eosin × 120)

Fig 2
Typical physaliforous cells (haematoxylin eosin × 120)

At autopsy the outward appearance was in no way abnormal When the skull was opened an extensive subarachnoid haemorrhage was found The haemorrhage was most pronounced on the anterior aspect of the pons in the pituitary fossa and around the pituitary gland but extended as a veil over the surface of both hemispheres On the clivus somewhat behind the pituitary fossa there was a lobulated whitish gelatinous tumour having a diameter of about 3 cm Its base measured 2 cm in diameter and consisted of irregular bony structure which appeared to extend down towards the nasopharynx Inspection of the nasopharynx revealed no abnormality There was a distinct impression of the tumour on the anterior aspect of

examination

On m croscopic study the tissue was found to be of a l ose structure and faintly staining some areas sh wing a cartilagenous appearance (Fig 1) Other areas were m re cellular and contained less ground substance The cells were reminiscent of embryon c cells having numerous minor cytoplasmic vacuoles typ cal physaliforous cells (Fig 2) Scattered among these cells there were fairly large tumour cells with a cyt plasm which was almost h mogeneous and nuclei with faintly staining chromatin and distinct nucleoli In the remainder of the cells the nuclei were more or less characterized by the cytoplasmic vacuolation In one place there were several collagen fibrils and a number of small vessels The cellular morphology was fairly varied but definite mitoses were not present The structure of the tumo r t ss e

a at t e base of the skull

CHORDOMAS

Chordoma is a fairly rare form of tumour It was described for the first time by *Luschka* in 1856 In Virchow s (1857) opinion these tumours issued from the cartilage between the sphenoidal and occipital bones In 1858 *Muller* advanced a presumption of its embryonic origin which was later confirmed by the experimental studies of *Ribberts* (1894) It was now realized that these tumours might also be of an extracranial position corresponding to the entire course of the notochord and that they were more often of a sacrococcygeal than an intracranial localization In 1935 *Mabrey* could collect 142 cases from the literature and add 8 of his own and in 1948 *Gentil & Coley* reported 128 published cases of sacrococcygeal chordomas adding 7 new cases of this special localization According to *Poppen & King* (1952) and *Crauford* (1958) a total of about 300 cases are on record

Only a few cases have been reported by Danish authors e g *Fabricius Moller* (1919) *Sorensen* (1937) *Moller* (1939) *Godtfredsen* 1944

in

m

As far as the intracranial chordomas are concerned the most common symptoms are increased intracranial pressure as well as signs of

Fig 1
Area showing a cartilaginous appearance (haematoxylin eosin × 120)

Fig 2
Typical physaliforous cells (haematoxylin eosin × 120)

epithelial appearance The cell limits are well marked, and the cells may be recognized by numerous clear vacuoles of varying size, separated by a faintly eosinophilic cytoplasm The vacuoles stain with Best's carmine which indicates a glycogen content The ground substance may vary somewhat in quantity, and the tumour is usually fairly avascular Vacuoles may be seen also in the ground substance The physaliferous cells may be arranged in cord shape or in an alveolar pattern In certain parts of the tumour, possibly its older part, the vacuoles may disappear, the cells assuming more stellate configurations which lend the appearance a certain resemblance to chondrosarcoma *Crawford* (1958) demonstrated that by certain staining methods, int al reticulin staining the ground substance of chordomas may be differentiated from that of chondrosarcomas, the former not taking this stain unlike the latter which becomes highly impregnated

The microscopic appearance, which for example seldom shows mitotic figures, does not warrant any conclusion regarding the degree of malignancy (*Godtfredsen* 1944, *Poppen & King* 1952) There may be tumours showing highly invasive growth whose microscopic structure does not exhibit the usual characteristics of malignant growth

Cause of Death in Intracranial Tumours

It is generally assumed and still stated in a few textbooks, that massive haemorrhage in intracranial tumours, leading to an apoplectiform syndrome, is common

However, this assumption is not supported by clinical pathological findings when disregarding the numerous minor haemorrhages in glioblastomas For instance, *Oldberg* (1933) found massive haemorrhage in only 31 (3 72 per cent) of 832 intracranial tumours of various types Only 7 of the patients had exhibited clinical symptoms characteristic of intracranial haemorrhage This corresponds to less than 1 per cent of all the studied cases Besides, among the cases without massive haemorrhage in the tumour, there were several running a course which was highly reminiscent of acute intracranial haemorrhage These observations have been supported by a number of workers, among others *Russel & Kershman* (1937) *Globus & Sapirstein* (1942) *Gross & Bender* (1948) *Manganiello* (1949), *Echols & Rehfeldt* (1950), and *Christensen* (1956)

It has been pointed out by *Poppen et al* (1952) that many cases of supratentorial tumours present haemorrhages in the pons which must be interpreted as being due to venous congestion The pathogenesis is, according to these authors that any increase in intracranial pressure will compromise the venous return causing an impaired drainage of the cerebrospinal fluid This sets up a vicious circle, and the intracranial pressure will increase even further For this reason the venous return is even more inhibited, inter alia through the supratentorial

neurological loss, depending upon the direction in which the tumour is growing In cases of vertebral chordomas, especially sacrococcygeal, the symptoms and signs are pain in the sacral region and not infrequently sphincter disturbances

Embryology

Chordomas arise from remnants of the embryonic notochord The notochord makes up the primitive axial skeleton of all vertebrates It is formed by cellular proliferation from the primitive streak and is situated between the neural groove and the entoderm A narrow cord of cells makes its way between the entodermal cells and thereby makes up part of the wall of the entodermal cavity These cells extend as far as the bucco pharyngeal membrane, while the most cranial part of the notochord ends at the sella turcica in the sphenoid bone According to *Hass* (1934) this gives the notochord an arcuate course, and owing to this arcuate course the notochord will be in several sites close to the dorsal part of the cartilaginous primitive skeleton One of these sites corresponds to the cartilaginous part of the dorsum sellae Gradually, the notochord becomes ensheated by a layer of cells from the mesoderm which later make up the vertebrae In the course of the formation of the vertebrae the notochord tissue is pressed into the intervertebral spaces where it contributes to the formation of the nucleus pulposus The notochord proper has usually completely regressed at birth but according to *Ribbert* (1904) and *Ormerod* (1960) remnants may be observed in the region of the clivus as well as in the intervertebral discs in 0 5–2 0 per cent of all cases at autopsy

For unknown reasons these remnants may suddenly start proliferating In the great majority of cases this growth is slow, resulting in small harmless processes looking like exotoses especially in the clivus More rarely, the growth is faster acquiring the nature of malignant tumour formation with invasion and destruction of bone in the surroundings The tumour has always issued from a site in the mid line, but the growth may take different directions which condition the variation in the character of the focal signs

Metastasization is relatively uncommon This applies particularly to distant metastases According to *Gentil & Coley* (1948) metastases have been observed in about 10 per cent of the cases On the other hand the rate of recurrence following surgical removal is very high, recurrence being rather the rule than the exception Another special feature is that despite its embryonic origin this tumour is extremely radio resistant If irradiation is to be of any use it has to be given in very high doses (*Rosenquist & Saltzman* 1959, Ormerod 1960)

Pathology

The gross appearance of chordomas is fairly uniform, whether they are of an intracranial or a vertebral situation The tumour is lobulated and of a soft, gelatinous consistence Its surface is smooth, greyish-white, and as a rule the tumour is enveloped in a thin, semi-transparent capsule Its size may range from 1 mm to 3 cm in diameter Far larger tumours have been reported, but they have usually been vertebral In more advanced cases the tumour is no longer well-defined, showing signs of invasive growth It often penetrates the dura, and destruction of the surrounding bony tissue is a common finding On section, the tissue is reddish-grey with minor haemorrhages On the other hand, calcifications and cystic degeneration of the tissue are rare

The microscopic appearance is rather varied The definite diagnosis is based on the presence of the so-called physaliferous cells which are pathognomonic of chordoma These are large, pale cells of a somewhat

epithelial appearance The cell limits are well marked, and the cells may be recognized by numerous clear vacuoles of varying size, separated by a faintly eosinophilic cytoplasm The vacuoles stain with Best's carmine which indicates a glycogen content The ground substance may vary somewhat in quantity, and the tumour is usually fairly avascular Vacuoles may be seen also in the ground substance The physaliferous cells may be arranged in cord shape or in an alveolar pattern In certain parts of the tumour, possibly its older part, the vacuoles may disappear, the cells assuming more stellate configurations which lend the appearance a certain resemblance to chondrosarcoma *Crawford* (1958) demonstrated that by certain staining methods, int al reticulin staining, the ground substance of chordomas may be differentiated from that of chondrosarcomas, the former not taking this stain unlike the latter which becomes highly impregnated

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vena cerebri magna system and through other venous systems draining inter alia the pons and mesencephalon. Thereby congestive venous haemorrhage in the named region may arise.

The pathogenesis of sudden exacerbation or death in cases of intracranial tumours without massive haemorrhage may be, in broad features, that progressing cerebral oedema and the growth of the tumour give rise to increased intracranial pressure and consequent venous congestive haemorrhages in the mesencephalon and the upper part of the pons around the vital centres in the reticular substance which govern respiration, pulse, and blood pressure. It is this type of congestive venous haemorrhage which gives rise to acute exacerbation of the condition, possibly with a fatal issue (*Poppen et al.* 1952, *Russel & Rubinstein* 1959).

DISCUSSION

In the present case of chordoma arising from the clivus the patient had not presented any actual symptoms before the fatal subarachnoid haemorrhage. Her only complaint was a bad taste in the mouth and bad breath. Signs of this kind have not been reported in the literature. *Poppen & King* (1952) have described a patient who had lost the sense of smell on one side, and one of *Ormerod's* (1960) patients had lost the sense of smell as well as the sense of taste. *Sorensen* (1937) has pointed out that the olfactory nerve is the only cerebral nerve which cannot be directly affected by a chordoma. If the patient has disturbances of smell, this must be due to the increased intracranial pressure. This is probably not the explanation in the present case, as the patient's complaints may be explained as a consequence of the detritus in the tonsillar crypts.

The author has not been able to find any reported case in which a chordoma has given rise to subarachnoid haemorrhage, and as already mentioned other types of intracranial tumours also seldom cause massive haemorrhage. In his comprehensive survey on subarachnoid haemorrhages of unusual aetiology *Walton* (1953) does not mention this type of tumour as an aetiological possibility. In one of *van Wagenen's* (1935) two cases of intracranial chordomas the patient suddenly died of subarachnoid haemorrhage, but autopsy showed that it was due to a ruptured basal aneurysm.

That this type of tumour may form the origin of massive haemorrhage into the subarachnoid space cannot be rejected. *Gardner & Turner* (1941) have reported that on section the tumour often shows minor haemorrhages in the tissue. In one of *Mauritzen et al.'s* (1959) patients who suddenly died, autopsy showed a major haemorrhage in the tumour. However, there was no haemorrhage in the surroundings. These findings make it very likely that chordomas may form the origin of fatal, massive subarachnoid haemorrhage, although this is rare as with other intracranial tumours.

Since no congenital malformations were found in the basal cerebral arteries and since the maximum haemorrhage in the present case was around the chordoma and around the pituitary gland, it seems predominantly likely that the fatal subarachnoid bleeding had originated in a chordoma on the clivus As such it appears to be the first published case of fatal subarachnoid haemorrhage having this unusual aetiology

SUMMARY

In connection with the description of fatal subarachnoid haemorrhage originating in a presumably malignant chordoma in the clivus of a 38 year old woman the embryology and pathology of chordoma are briefly reviewed

The present case appears to be the first published case of fatal subarachnoid haemorrhage of this aetiology

It is pointed out that intracranial tumours on the whole very seldom give rise to massive intracranial haemorrhages

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EFFECT OF ENDOTOXIN ON MAST CELLS AND THE EXTENSION OF THE LOCAL SHWARTZMAN REACTION TO THE HAMSTER

By

GUNNAR T GUSTAFSON and STIG CRONBERG

Received 5 1 63

The fundamental mechanism of the biological activities of bacterial endotoxins is obscure Since many of their effects can be imitated by antigen antibody complexes it has been suggested that endotoxins may be toxins only in the sense that tuberculin and other antigens are toxic for the hypersensitive animal (*Stetson* 1955 *Robbins & Stetson* 1959) Though it has not been possible to classify cellular and humoral reactivity to endotoxins as an immediate type of hypersensitivity the release of histamine and 5 hydroxytryptamine following endotoxin injection implies that these agents are involved in the development of endotoxin reactions (*Lecomte* 1956 *Weil & Spink* 1957 *Thomas, Zweifach & Benacerraf* 1957 *Spink* 1960 *Greisman* 1959 *Hinshaw et al* 1960 *Spink* 1962) Since mast cells are degranulated and release heparin histamine and in some species 5 hydroxytryptamine as a consequence of antigen antibody reactions (*Wegelius et al* 1955 *Carter et al* 1957 *Mota* 1958 *Boreus* 1960) an 1 h agents such as peptone 48 80 and 938 *Jaques & Waters* 1941 *Riley* 957, *Gustafsson B F & Cronberg* 19) it has been suggested that mast cells may be involved in the endotoxin reactions (*Spink* 1960)

The studies reported here are concerned with the mast cell alterations after local injection of endotoxin after local

MATERIALS AND METHODS

Hamsters Most experiments were pe f ing from 80 to 110 g They w and water ad lib Rabbits W were used for some local Shwa

Endotoxins E coli lipopolysaccharide Difco O 226 B was used for the injections

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in the cheek pouch mucosa and for the intravenous injections In some experiments an endotoxin prepared from oral fusobacteria by the phenol water method mainly as described by *Westphal et al* (1952) was used In all experiments one cheek pouch was injected with endotoxin and the contralateral pouch was given a control injec ti n with sodium chloride solution

Histopathological technique After the macroscopic reaction had been noted the cheek pouches were excised and a strip was mounted on a glass This was fixed in absolute alcohol and stained for 24 hours in 2 per cent toluidine blue and 40 per

clearing in xylene
lso fixed in a solu
nt acetic acid for 24
ined with toluidine

blue in alcoholic and aqueous solutions and also with haematoxylin and eosin

In some experiments white rats and hamsters were injected intraperitoneally and spreads of the mesentery were prepared and counts of the mast cells in the mesen tery and differential counts of the cells in the peritoneal fluid were made in the

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s with the
disrupted

EXPERIMENTAL

Effect of Local Injection of Endotoxin

Necropsy of 7 hamsters sacrificed 1–3 hours after intraperitoneal in jection of endotoxin showed an increased amount of fluid within the peritoneal cavity and slight dilatation of the vessels

The mast cells in the peritoneal fluid and in the mesentery of the animals were unaffected A moderate neutrophilic leucocytosis was ob served (*Table 1*) Also in the hamster cheek pouch the mast cells were intact and ... ction of

rats

Effect in Local Shwartzman Reaction

In one series of 19 hamsters the cheek pouch on one side was injected with coli endotoxin 20 hours later coli endotoxin was given intra venously and the hamsters were sacrificed 0 5 to 24 hours later (*Table 2*)

The first objective signs of the reactions some petechiae, were ob served 1½ to 2 hours after the intravenous injection After 3 hours the reaction obviously corresponded well to the classical local Shwartzman reaction in the rabbit skin consisting of submucosal haemorrhage and oedema (Fig 2) ... more or less necro... pouch It was pos... the E coli endotoxin by Fusobacteria endotoxin in the preparative injection or in the intravenous injection or in both

Histologically the classical features consisting of accumulations of leucocytes within the blood vessels were apparent already within the

TABLE 1

Effect of E coli Endotoxin in the Peritoneal Cavity of Hamster

Substance tested and mode of administration	Duration after the last injection (hours)	Dosis	Number of hamsters	Mast cells totally	Types of mast cells				Types of cells in periton cavity				
					A	B	C	D	M	L	G	N	L
E coli endotoxin intraperitoneally	1–3	500 µg	7	175	80	18	2	0	5	5	2	50	38
E coli endotoxin intraperitoneally + after 24 hours intravenously E coli endotoxin	4–6	500 µg 500 µg	5	101	77	23	0	0	4	2	1	33	60
E coli endotoxin Intravenously + 1 hour later epinephrine	24	500 µg 200 µg	2	145	77	23	0	0	0	0	1	43	56
Intraperitoneal inj of sodium chloride	1	0 5 ml	4	179	56	38	6	0	8	2	4	86	0

Symbols

Different types of mast cells in the mesentery expressed in percentages

A = Normal mast cells with no ejected granules
B = Cells with 1–10 ejected granules
C = Cells surrounded by granules but with the central part intact
D = Totally disrupted mast cells with the granules evenly scattered

Types of cells in the peritoneal fluid expressed in percentages

M = Mast cells
E = Eosinophil leucocytes
G = Mononuclears with phagocytized granules
N = Mononuclears without phagocytized granules
L = Neutrophil leucocytes

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The mast ce ...
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... was infiltrated and often the tissue became more or less necrotic No reactions were observed in the control cheek pouch It was possible to replace the E coli endotoxin by Fusobacteria endotoxin in the preparative injection or in the intravenous injection or in both

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first hour of the intravenous injection (Fig 1) Later thrombosis of the vessels and haemorrhage were observed Necrosis of epithelial and other tissue cells were often noted The mast cells were mostly unaffected (Figs 3, 7) In later stages, however, they were often disrupted in certain areas (Fig 4) where the other cells also showed signs of necrosis with changed stainability

TABLE 3

Effect of Epinephrine on Mast Cells in Cheek Pouch of Hamsters Pretreated with Endotoxin

Intravenous inj of endotoxin	Interval between inj of 200 g epinephrine and sacrifice (hours)	Reaction noted in cheek pouch		Mast cell disruption in cheek pouch	
		Tested	Control	Teste l	Control
E coli 500 g	3	+	—	++	—
"	24	+	—	+++	—
	24	+	—	+++	—
"	24	+	—	++	—
"	24	+	—	++	—
	24	+	—	+++	—
"	24	+	—	++	
	24	+	—	+++	—
	24	+	—	++	—

Hamsters were injected intravenously with endotoxin and one hour later followed by an eninephrine injection in the 'test' pouch and 0 9 per cent sodium chloride solution in the 'control" pouch

The reactions are graded in the same way as in Table 2

The gross reaction consisted of desquamation of the mucosal epithelium and there were no signs of haemorrhagic necrosis

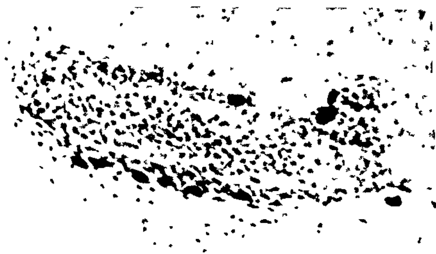


Fig 1

Blood vessel packed with neutrophil leucocytes one hour after intravenous endotoxin injection in a hamster cheek pouch pretreated with endotoxins The mast cells are intact

TABLE 2

Effect of Local Shwartzman Reaction on Mast Cells in Hamster Cheek Pouch

Preparative local injection of endotoxin Material dose (g)	Interval between intravenous inj and sacrifice (hours)	Reactions noted in cheek pouch		Mast cell disruption in cheek pouch	
		Tested	Control	Tested	Control
E. coli 500	0.5	—	—	—	—
" "	1	—	—	—	—
" 200	1	—	—	—	—
" 500	1 5	+	—	—	—
" "	2	+	—	—	—
" "	2 5	++	—	—	—
" "	3	++	—	—	—
" "	3	++	—	—	—
" "	4	+	—	—	—
" "	4	+	—	—	—
" "	4	++	—	—	—
" "	4	++	—	—	—
" "	4	+++	—	—	—
" "	4 5	+++	—	—	—
" "	5	+++	—	+	—
" "	6	++	—	+	—
" "	6	+++	—	++	+
" "	12	++	—	—	—
" "	24	+++	—	+	—
" ,	24	+	—	—	+
Fusobacteria 500	6	++	—	+	+
" "	6	++	—	—	—
" "	6	++	—	+	—
" "	6	++	—	+	—
" "	6	++	—	+	—
, ,	6	++	—	+	—
" ,	6	+++	—	+	—
,	6	+++	—	+	—

All hamster were injected in the tested pouch with the endotoxin solution and in the control pouch with sodium chloride solution 20–24 hours later the animal received 200 500 E coli endotoxin intravenously

Explanation of the different grades of reactions

Local Shwartzman reaction in the cheek pouch

— = No gross reaction
+ = Dilated vessels and slight oedema, sometimes petechiae
++ = Submucosal haemorrhage and induration
+++ = Intense submucosal haemorrhage, induration and often necrosis of the pouch mucosa

Mast cell reaction

— = Quite normal mast cells with practically no disrupted cells
+ = Disrupted cells can be found in a limited area
++ = Disruption or disappearance of mast cell is evident in large areas, but most of the cells are still unaffected
+++ = Most cells are disrupted

first hour of the intravenous injection (Fig 1) Later thrombosis of the vessels and *haemorrhage* were *observed* *Necro* is of epithelial and other tissue cells were often noted The mast cells were mostly unaffected (Figs 3 7) In later stages, however, they were often disrupted in certain areas (Fig 4) where the other cells also showed signs of necrosis with changed stainability

TABLE 3

Effect of Epinephrine on Mast Cells in Cheek Pouch of Hamsters Pretreated with Endotoxin

Intravenous inj of endotoxin	Interval between inj of 200 g epinephrine and sacrifice (hours)	Reaction note 1 in cheek pouch		Mast cell disruption in cheek pouch	
		Tested	Control	Tested	Control
E coli 500 g	3	+	—	++	—
	24	+	—	+++	—
	24	+	—	+++	—
	24	+	—	++	—
	24	+	—	++	
	24	+	—	+++	
	24	+	—	++	
	24	+	—	+++	—
	24	+	—	++	—

Hamsters were injected intravenously with endotoxin and one hour later followed by an epinephrine injection in the "test" pouch and 0 9 per cent sodium chloride solution in the "control" pouch

The reactions are graded in the same way as in Table 2

The gross reaction consisted of desquamation of the mucosal epithelium and there were no signs of haemorrhagic necrosis



Fig 1

Blood vessel packed with neutrophil leucocytes one hour after intravenous endotoxin injection in a hamster cheek pouch pretreated with endotoxins The mast cells are intact

TABLE 2

Effect of Local Shwartzman Reaction on Mast Cells in Hamster Cheek Pouch

Preparative local injection of endotoxin Material dose (g)	Interval between intravenous inj and sacrifice (hours)	Reactions noted in cheek pouch		Mast cell disruption in cheek pouch	
		Tested	Control	Tested	Control
E coli 500	0 5	—	—	—	—
" "	1	—	—	—	—
" 200	1	—	—	—	—
, 500	1 5	+	—	—	—
" "	2	+	—	—	—
" "	2 5	++	—	—	—
" "	3	++	—	—	—
" "	3	++	—	—	—
" "	4	+	—	—	—
" "	4	+	—	—	—
" "	4	++	—	—	—
" "	4	++	—	—	—
" "	4	+++	—	—	—
" "	4 5	+++	—	—	—
" "	5	+++	—	+	—
" "	6	++	—	+	—
" "	6	+++	—	++	+
" "	12	++	—	—	—
" ,	24	+++	—	+	—
" "	24	+	—	—	+
Fusobacteria 500	6	++	—	+	+
" "	6	++	—	—	—
" "	6	++	—	+	—
" "	6	++	—	+	—
" "	6	++	—	+	—
" "	6	++	—	+	—
, "	6	+++	—	+	—
" "	6	+++	—	+	—

All hamster were injected in the tested pouch with the endotoxin solution and in the control pouch with sodium chloride solution 20 24 hours later the animal received 200–500 E coli endotoxin intravenously

Explanation of the different grades of reactions

Local Shwartzman reaction in the cheek pouch

— = No gross reaction
+ = Dilated vessels and slight oedema sometimes petechiae
++ = Submucosal haemorrhage and induration
+++ = Intense submucosal haemorrhage, induration and often necrosis of the pouch mucosa

Mast cell reaction

[illegible] tically no disrupted cells
[illegible] ited area
+ [illegible] cell is evident in large areas, but

+++ = Most cells are disrupted

Similar results were obtained in rabbits, but because of the low frequency of mast cells in this species the result was not so impressive as in hamster

No reaction comparable to local Shwartzman reaction could be elicited in the peritoneal cavity and the mast cells appeared normal (Table 1)

Elicitation of Local Shwartzman Reaction in Hamster Cheek Pouch Depleted of Mast Cells

In order to ascertain whether the local Shwartzman reaction could be elicited in the abscence of mast cells nine hamsters were given 3 5 ml distilled water in each cheek pouch, it being known that distilled water has the potency to deplete mast cells by disruption (*Fawcett* 1955) One day later a local injection of 500 µg Γ coli endotoxin was given in one of the cheek pouches and the contralateral served as control and was injected with a sodium chloride solution When an intravenous injection of endotoxin was given 24 hours later a Shwartzman reaction of the same extension and chronological order as described above was elicited in the endotoxin treated cheek pouch (Fig 5), but not in the control pouch The histological picture revealed no mast cells in the center of the cheek pouch, where the Shwartzman reaction had been elicited (Fig 6) Only in the periphery of the pouch could mast cells be found

Protamine chloride and toluidine blue were also used in six hamsters in order to destroy the mast cells, but the large doses necessary to produce a more extreme destruction of the mast cells had an necrotizing effect which made the result more difficult to interpret However, it seemed to be of the same type as those observed after injection of distilled water Also the control pouch which had received protamine chloride but not endotoxin, showed a reaction *similar to that* in the endotoxin-injected pouch, but weaker

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Fig 2–7

Fig 2 Shwartzman reaction in the hamster cheek pouch
Fig 3 Shwartzman reaction in the hamster cheek pouch showing intact mast cells

nast

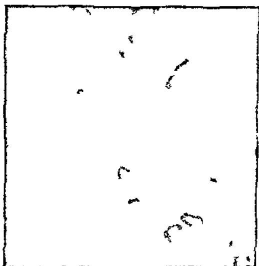
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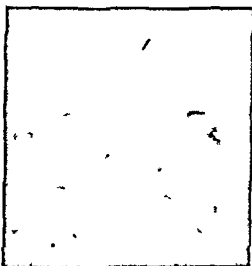
Fig 7 Shwartzman reaction in the hamster cheek pouch showing intact mast cells despite intense haemorrhage

The histological preparations have been stained with toluidine blue in alcoholic solution Fig 1 is a section The rest are from whole mounts


2


5


3


6


4


7

Similar results were obtained in rabbits, but because of the low frequency of mast cells in this species the result was not so impressive as in hamster

No reaction comparable to local Shwartzman reaction could be elicited in the peritoneal cavity and the mast cells appeared normal (Table 1)

Elicitation of Local Shwartzman Reaction in Hamster Cheek Pouch Depleted of Mast Cells

In order to ascertain whether the local Shwartzman reaction could be elicited in the absence of mast cells nine hamsters were given 3 5 ml distilled water in each cheek pouch, it being known that distilled water has the potency to deplete mast cells by disruption (*Fawcett* 1955) One day later a local injection of 500 µg E coli endotoxin was given in one of the cheek pouches and the contralateral served as control and was injected with a sodium chloride solution When an intravenous injection of endotoxin was given 24 hours later a Shwartzman reaction of the same extension and chronological order as described above was elicited in the endotoxin treated cheek pouch (Fig 5), but not in the control pouch The histological picture revealed no mast cells in the center of the cheek pouch, where the Shwartzman reaction had been elicited (Fig 6) Only in the periphery of the pouch could mast cells be found

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---

Fig 2 7

Fig 2 Shwartzman reaction in the hamster cheek pouch
Fig 3 Shwartzman reaction in the hamster cheek pouch showing intact mast cells
Fig 4 Shwartzman reaction in the hamster cheek pouch showing disrupted mast cells in a necrotic area
[illegible] tilled water [illegible] treatment with [illegible] have been de[illegible]
Fig 7 Shwartzman reaction in the hamster cheek pouch showing intact mast cells despite intense haemorrhage

The histological preparations have been stained with toluidine blue in alcoholic solution. Fig 1 is a section The rest are from whole mounts

Effect of Epinephrine in the Presence of Endotoxin.

The typical haemorrhagic necrosis as a result of the combined effect of endotoxin and epinephrine in rabbits was found to result in the disappearance of mast cells in some areas only. The relative frequency of mast cells, however, was difficult to estimate because of the uneven distribution and low number of these cells in the rabbit.

An intravenous injection of 500 μg E coli endotoxin followed by an injection of 200 μg epinephrine in the cheek pouch of hamster produced signs of necrosis The cheek pouch was slightly reddened and edematous, and the epithelium had a tendency to loosen. It should be stressed that the reaction was not like that produced in the skin of rabbits. Most of the mast cells were disrupted (Table 3). This could be seen already after three hours The appearance after one day, however, was more like that of a recent disruption with extracellular granules and very little phagocytosis The mast cell disruption was always associated with necrosis of the epithelium. Epinephrine alone produced similar changes although they seemed to be slightly weaker

DISCUSSION

The animal most used in the study of the local reaction to endotoxin is the rabbit and the local Shwartzman reaction was given its original definition in this species (*Shwartzman* 1928) However, in the investigation of mast cells the rabbit has disadvantages as a test animal The tissue contains relatively few mast cells, and they are very sensitive to various treatments In addition, the bulk of histamine has been referred to the platelets in this species (*Humphrey* 1959) Though attempts by Shwartzman and others to extend the local Shwartzman reaction to other animals were generally unsuccessful, it has been seen in guinea pigs (*Gratia & Linz* 1931) and under special circumstances in mice (*Stone & Freund* 1956, *Kelly et al* 1957, *Arndt & Schneider* 1960) Since mast cells are more numerous in rats and hamsters, these animals were studied for their capacity to produce the local Shwartzman reaction While the experiments were unsuccessful in the rat a very strong and typical reaction was obtained in the hamster cheek pouch Since this demonstrated a substantial endotoxin reaction and since this tissue is rich in mast cells and whole mounts and histological sections could easily be prepared, the hamster cheek pouch was selected for the present study Nothing resembling the Shwartzman reaction was observed in the mesentery

Like *Antweiler & Hirsch* (1961) we observed no direct disruptive effect of endotoxins on the mast cells In *in vitro* studies *Asboe-Hansen & Glick* (1958) found endotoxin to have no effect on the mast cells in rat peritoneal fluid either However, when the rats were pretreated systemically with E coli endotoxin, these authors found that hyaluronidase degranulated more mast cells than in untreated animals This

has been taken as support for the view that endotoxin can influence the mast cells and therefore be of importance in the pathogenesis of gram negative infections (*Spink* 1960) However, in the experiments of Asboe-Hansen and Glick the mast cells were stained supravitally with toluidine blue, which disrupts mast cells *in vivo* (*Gustafsson, B E & Cronberg* 1959)

In the local Shwartzman reaction we observed no disruption of mast cells in the first 4 hours after the eliciting dose After this period local mast cell destruction could be observed, but then the other tissue cells were also necrotic This mast cell destruction was therefore taken as a secondary effect

These results are in accordance with those of *Miles* (1960), who produced bacterial inflammation in the peritoneal cavity of cats by a variety of organisms including Gram negative bacteria without histamine release or mast cell degranulation In personal pilot experiments viable coli bacteria injected intraperitoneally into rats did not disrupt mast cells

The fact that the local Shwartzman reaction could be elicited in tissue devoid of mast cells further supports the concept that there is no intimate relations between these cells and the endotoxin reactions studied The apparent lack of involvement of mast cells in the local Shwartzman reaction also indicates that another type of mediator of local tissue damage than those associated with the release of histamine from mast cells, may be implicated in these vessel wall injuries Turning to the systemic effects of endotoxin, the results seem compatible with the view of *Delaunay et al* (1948), *Zweifach & Thomas* (1957) and *Halpern* (1962), that these effects are more like the traumatic shock, while anaphylactic shock bears a closer resemblance to histamine intoxication In the epinephrine experiments in hamsters necrosis without haemorrhage was produced in contrast to the skin reactions in the rabbit As epinephrine alone had a necrotizing effect in the hamster cheek pouch, the additional effect of endotoxin is difficult to interpret Also in these reactions, however, the mast cell destruction seemed to be secondary to the necrosis of the tissue

SUMMARY

The effect of endotoxin on mast cells was studied after local injection in the local Shwartzman reaction and after local injection of epinephrine in endotoxin treated animals Local injection was found to have no effect on mast cells in the peritoneal cavity of hamster, in the rat or in the hamster cheek pouch The local Shwartzman reaction was demonstrated in the hamster cheek pouch This reaction was considered equivalent to the local Shwartzman reaction encountered in the rabbit as it conformed to all the operational and histopathological characteristics of the classical reaction, including elicitation by various

endotoxins as well as heterologous preparation and provocation In these reactions the mast cells disrupted to some degree, but this effect seemed secondary to the haemorrhagic necrosis In the hamster cheek pouch depleted of mast cells by distilled water, the local Shwartzman reaction could still be produced It is concluded that mast cell alterations do not seem to be an essential or a primary effect in endotoxin reactions

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With the aid of x-ray microscopy the present investigation has been made to study the reaction of the mineralized part of the bone tissue of rats at different intervals after drinking fluoridated water of various concentration

MATERIAL AND METHODS

642 specimens from 167 rats of both sexes of three generations of the Sprague Dawley strain have been studied The rats have been divided into six groups including a control group of 35 rats The rats have been given distilled water containing fluorine in concentrations 0 1 5 10 20 and 40 ppm (part per million) respectively All the rats have been offered the water since they were born (even the *mothers were given the same water) and killed by decapitation at different intervals* thus giving an age varying from three months to three years

The average consumption of a rat was 27 ml ± 3 ml per 24 hours no significant difference bet... including th... Uppsala) T... ashed at 560... Beckman ph... a known am... fresh bread... proximate bread consumption of 40g day the total mean intake of fluoride (water + bread) by each rat will then be

Group I	1 1 mg F/day
II	0 7
III	0 4
IV	0 3
V	0 2
control	0 1

From each rat longitudinal sections have been made from the femur lower jaw right pelvis and from the vertebrae In cases where the x ray microscopical picture was equivocal extra specimens were taken *from the left side* Results from specimens whose microradiograms were still difficult to interpret were discarded

Plane parallel ground section with a thickness of about 75 µ for contact microradiography and about 20 µ for x ray fluorescence analyses were prepared by the method of *Hallen & Rockert* (1960)

X ray absorption studies have been made by contact microradiography using a Machlett tube OEG 50 A with Cu anode 1 mm Be filter 1 5 mm focal spot at 10 kV and 50 mA The film used was ... retical discussions ... *Lindstrom* ... 1946) A the micro ... 1 2 4 areas repre ... White under a 1 ... mined ... distri ... the aid of the x ray with a Cosslett Nixon ... two deflection coils ... of a specimen holder ... aperture resting on a 9 µ Cu anode The fluorescent radiation was generated in the specimen by the Cu rad... fluorescent ... d was re ... *Ca ana* ... the exposure time for each ... was two minutes Only relative values have been recorded and these are expressed as the ratio Ca/Fe The method has been described in detail (*Rockert* 1962 *Long & Rockert* 1962) Correction factors for specimen thickness are given by *Long* (1958)

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X-RAY ABSORPTION AND X-RAY FLUORESCENCE MICRO-ANALYSES OF MINERALIZED TISSUE OF RATS WHICH HAVE INGESTED FLURIODATED WATER

By

HANS ROCKERT

Received 2 II 63

Fluorine as a possible cause of skeletal changes has been discussed in connection with the ingestion of fluoride containing water by many authors

Roholm (1937) studied changes in the bone of Danish cryolite workers who had been exposed to dust containing fluoride Radiography showed various degree of osteosclerosis beginning in the pelvis and vertebrae More severe cases with gross changes in the ribs, sternum and in the limbs also, have been reported (*Sing et al* 1962)

In view of a reported bone resorption effect of fluorine, it has been tested therapeutically in cases where the skeletal mass is reduced, *Rich & Ensinck* (1961) For an extensive survey of literature on *human* material after fluoride intake see *Largent* (1961)

Experiments on *animals* have shown that fluorine is mainly incorporated and accumulated in the bone tissue, (*Blayney, Bowers & Zimmerman* 1962) Active areas of bone incorporated more fluorine than static regions, *Miller & Phillips* (1956), *Suttie & Phillips* (1959) and *Weidmann & Weatherell* (1959), *Ohnell et al* (1936) At a certain level of fluorine intake the metabolism of the bone tissue is altered and the balance between osteoblastic and osteoclastic activity may be upset

Fluoride accumulated in skeltal tissue in proportion to the degree of exposure, *Largent* (1961) *Phillips, Greenwood, Hobbs & Huffman* (1955) noticed that during the accumulation of fluorides the bones "may become varying degrees less compact, more porus, and softer than normal Some fluoridated bones will crush under pressure before they will break sharply"

Clinical, *radiological*, chemical or histological investigations have failed to detect early bone changes in x-rays see, for example, *Largent*, *Machle & Ferneau* (1943) X-ray microscopy provides a quantitative method to study the degree and pattern of mineralization and correlate it with the micromorphology of the bone tissue

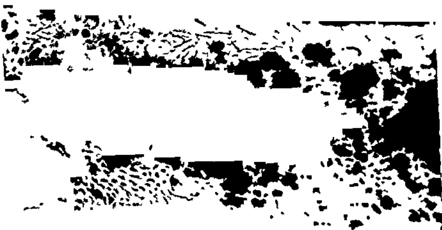


Fig 2
Microradiogram of the right femur of a 2 year old rat from group 1

Fig 3 Fig 4

Microradiogram of part of the incisor and the alveolar bone from the same rat as in fig 1 Note the globular dentin in fig 4



Fig 1
Microradiogram of the lower jaw from a 2 year old rat of group I

RESULTS

The bone tissue from rats of group I and II show on the microradiograms first of all osteosclerosis, mainly visible in the vertebrae (*Rockert & Sunzel* 1960) This osteosclerosis has been detected after 9-12 months After a year small demineralized areas appear. At an early stage these pathological changes are apparent in the femur and the jaw In several cases from group I and II the bone tissue show advanced pathological changes, mainly large demineralized areas (Fig 1 and 2)

The lower incisors of the 6-months rats show an increased amount of interglobular dentin In rats more than a year old the dentin shows globular degeneration with a widened pulp, (Fig 3) No osteosclerosis is seen in the groups III, IV and V The same type of resorption cavities however, were seen in these groups The number of specimens of groups *III and IV was unfortunately low Generally, however, the cavities in* the groups III, IV and V were fewer No resorption cavities were seen in the control group See Table 1

In cases without microscopical changes x-ray fluorescense microanalyses failed to show any significant difference in the Ca/Fe ratio in groups III, IV and V compared to the control group, *i e* no diffuse demineralization occurred

In the microradiograms no significant differences in the mineralization between the generations or between the sexes has been found

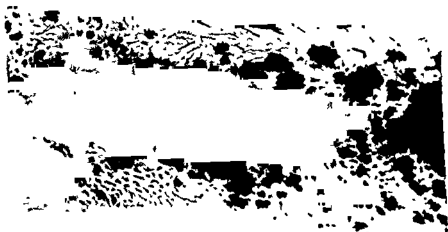


Fig 2
Microradiogram of the right femur of a 2 year old rat from group 1

Fig 3 *Fig 4*

Microradiogram of part of the incisor and the alveolar bone from the same rat as in fig 1 Note the globular dentin in fig 4

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EARLY CHANGES IN ALLERGIC ENCEPHALOMYELITIS IN RABBITS

By

KARL-ERIK ÅSTRÖM

Received 20 II 63

Experimental allergic encephalomyelitis (EAE) appears to be based upon the so called "delayed" type of hypersensitivity (*Waksman* 1959). In the elucidation of the events, which lead to actual lesions formation, the problem of early changes is of great interest (*Condie & Good* 1959, *Alvord, Magee, Kies & Goldstein* 1959, *Alvord* 1959, *Field* 1961). The histology of early lesions in guinea pigs and rabbits has recently been described in detail by *Waksman & Adams* (1962). They found that the lesions appear to start with perivenous infiltration by mononuclear cells, and concluded that myelin breakdown is associated with this phenomenon. The results of the present work, which represents a similar study on rabbits, are in agreement with their findings. A summary will therefore be published mainly in order to confirm these, and to add a few further descriptive details.

METHODS

Rabbits weighing 2.5–3.0 kg were inoculated with a mixture of bovine spinal cord (6.5 gm), Difco's Bacto Adjuvant Complete Freund (10 ml) and additional heat killed tubercle bacilli (23 mg). An injection of 0.05–0.1 ml was administered intradermally into a single toepad of each leg. The animals were then observed daily and killed after 7–14 days, before or shortly after the appearance of symptoms (see the Table). After examination of the cerebro-spinal fluid, the brain, spinal cord, roots and sensory ganglia were removed and fixed in formalin. Representative pieces were embedded in paraffin and stained according to conventional histologic methods.

RESULTS

Histology: Sensory Ganglia, Spinal Nerve Roots, Meninges and Parenchyma of the Spinal Cord

Lesions (Table) were seen in the sensory ganglia of all the 31 animals investigated. In the earliest phase, lymphocytes appeared between the nerve cell bodies (Fig. 1). Then the inflammatory cells increased in

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FROM THE DEPARTMENT OF PATHOLOGY II KAROLINSKA INSTITUTET
SABBATSBERGS SJUKHUS STOCKHOLM SWEDEN
(HEAD PROF NILS RINGERTZ M D)

EARLY CHANGES IN ALLERGIC ENCEPHALOMYELITIS IN RABBITS

By

KARL-ERIK ÅSTRÖM

Received 20 II 63

Experimental allergic encephalomyelitis (EAE) appears to be based upon the so called "delayed" type of hypersensitivity (*Waksman* 1959). In the elucidation of the events which lead to actual lesions formation, the problem of early changes is of great interest (*Condie & Good* 1959 *Alvord*, *Magee*, *Kies & Goldstein* 1959, *Alvord* 1959, *Field* 1961). The histology of early lesions in guinea pigs and rabbits has recently been described in detail by *Waksman & Adams* (1962). They found that the lesions appear to start with perivenous infiltration by mononuclear cells and concluded that myelin breakdown is associated with this phenomenon. The results of the present work, which represents a similar study on rabbits, are in agreement with their findings. A summary will therefore be published mainly in order to confirm these, and to add a few further descriptive details.

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Histology Sensory Ganglia Spinal Nerve Roots, Meninges and Parenchyma of the Spinal Cord

Lesions (Table) were seen in the sensory ganglia of all the 31 animals investigated. In the earliest phase, lymphocytes appeared between the nerve cell bodies (Fig 1). Then the inflammatory cells increased in

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TABLE

Clinical Histological and Cerebrosp

Symptoms		Day of necropsy	Histolog	
			Sp	
Time of onset (days)	Degree on day of necropsy		Sensory ganglia	Leptomeninges
	—	7	++	+
	—	7	++	+
	—	7	+	+
	—	8	+	+
	—	8	+	+
8	+	8	+++	+++
8	+	8	+++	++
8	++	8	+++	+++
	—	9	(+)	(+)
	—	9	++	(+)
	—	9	+	(+)
9	+	9	+++	++
8	++	9	+++	+++
	—	10	(+)	(+)
	—	10	+++	+
	—	10	++	(+)
10	+	10	+++	+++
10	+	10	+++	++
10	+	10	+++	++
10	+	10	+++	++
10	+	10	+++	++
	—	11	++	(+)
	—	11	+++	++
10	++	11	+++	+++
10	++	11	+++	++
	—	12	(+)	(+)
	—	12	+++	++
	—	12	+++	++
	—	13	++	+
	—	14	+	(+)
	—	14	++	+

Symptoms and lesions graded from — to +++ All animals with encephalitozoo

number, forming larger, partly confluent infiltrates, usually around small veins The cell picture was always dominated by lymphocytes, monocytes and macrophages, although polymorphonuclear leucocytes (mostly eosinophils) and plasma cells also occurred regularly in the larger lesions The infiltrates appeared mainly in the peripheral parts of the ganglia, which contain nerve cell bodies Only in the more severe cases were inflammatory cells seen also between the fibre tracts centrally, and then, usually, only in small numbers In one case with pronounced symptoms, small necrotic areas and haemorrhages were noted in one ganglion

Degeneration of myelinated fibres was never observed in the smallest lesions in the ganglia it occurred only in relation to the larger cell infiltrates It was remarkable that the nerve cell bodies usually appeared

luid Findings in Necropsied Rabbits

ndings				Cerebrospinal fluid	
ord	Brain				
Parenchyma	Cerebellum	pons Cerebrum		Cells mm^3	Total protein (mg 100 ml)
	—		—	2	14
(+)	—		—	75	130
—	—		—	4	16
—	—		—	18	56
	—		—	4	17
++	+		(+)	300	250
+	+		+	81?	coag
+	—		—	116?	coag
—	—		—	6	16
—	—		—	9	no obs
—	—		—	18	18
+	—		—	140	330
+++	—		—	1200?	coag
—	—		—	12	20
—	—		—	33	77
—	—		(+)	no obs	no obs
+	+		+	940	sl bloody
+	—		—	280	230
++	+		(+)	330	180
+	—		—	97	150
+	-		+	55	85
—	—		—	14	16
—	—		—	205	350
++	+		—	no obs	no obs
+	+		—	no obs	no obs
—	—		—	26	16
++	—		—	230	125
+	—		(+)	170	63
+			—	55	33
—	—		—	8	14
—	—		—	90	no obs

infection excluded

unaffected, even when they were surrounded by large numbers of inflammatory cells

The subarachnoid space was invariably found to contain inflammatory cells When the number of cells was small, these consisted exclusively of lymphocytes and monocytes The larger collections were made up mainly of lymphocytes and macrophages, but frequently some eosinophils, neutrophils and plasmacells were also pr[illegible]

[illegible] in many cases The lesions, when present in an animal, were always less severe than those in its

TABLE

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8	+	8	+++	++
8	++	8	+++	+++
	—	9	(+)	(+)
	—	9	++	(+)
	—	9	+	(+)
9	+	9	+++	++
8	++	9	+++	+++
	—	10	(+)	(+)
	—	10	+++	+
	—	10	++	(+)
10	+	10	+++	+++
10	+	10	+++	++
10	+	10	+++	++
10	+	10	+++	++
10	+	10	+++	++
	—	11	++	(+)
	—	11	+++	++
10	++	11	+++	+++
10	++	11	+++	++
	—	12	(+)	(+)
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	—	12	+++	++
	—	13	++	+
	—	14	+	(+)
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Symptoms

Signs of disease were seen 8 days or later, after the injection of the antigen (Table) the first manifestations being ataxia and weakness of the hind legs followed by similar symptoms in the forelegs general weakness lack of appetite etc There were as a rule no evident cerebral symptoms Clinical signs were lacking in several cases with pronounced lesions especially in the spinal ganglia

Cerebrospinal Fluid

At least some cells were constantly found in the cerebrospinal fluid (Table) These cells were always mononuclear except in a few cases with high pleocytosis where a small number of polymorphonuclear leucocytes were also seen A gross correlation between the number of cells and clinical codition could be established The number was usually below 90 mm³ in animals without signs of disease and above 90 mm³ in those with such signs The cell number was low in a few animals with severe lesions in the sensory ganglia but without symptoms The spinal fluid protein was largely correlated to the number of cells

DISCUSSION

The lesions of EAE in this series were seen in an early phase of the disease i e before or shortly after the onset of symptoms The gross pattern of these early lesions was rather uniform they affected mainly the spinal cord and there in the following order sensory ganglia leptomeninges and spinal cord parenchyma This pattern seems to be determined partly by the distribution of the veins (*Waksman* 1960)

The development of the individual lesions – as interpreted from the histological material was also rather uniform Collections of lymphocytes and monocytes in the above mentioned sites invariably represented the first signs of disease and the destruction of myelin occurred later Consequently these mononuclear cells seem to be of primary significance for the formation of lesions This notion is of special interest in view of the role of sensitized lymphocytes for passive transfer of EAE (*Paterson* 1961 *Åstrom & Waksman* 1962) Polymorphonuclear cells and plasma cells exudation of plasma protein etc also appeared later and can therefore be regarded as secondary phenomena The observations and conclusions referred to in this section are in agreement with those of *Waksman & Adams* (1962)

In order to study true early lesions of EAE it seems most expedient to sacrifice the animals before the appearance of clinical manifestations

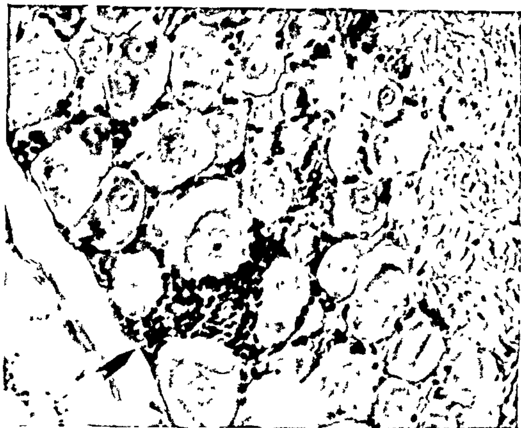


Fig 1

Rabbit No symptoms Killed at 7 days Early lesion of mononuclear cells between nerve cells in dorsal root ganglion (arrow) Haematoxylin and eosin × 360

sensory ganglia and meninges, and consisted of perivascular cuffs of round cells or of subpial foci of myelin degeneration or of both The perivascular cell collections extended from the surface into the white matter at varying depths The myelinated fibres in the surrounding zones were sometimes, but not always, affected The subpial foci of demyelination were usually situated beneath patches of inflammatory cells in the overlying meninges They could become confluent at the surface, but did not extend very deeply Areas with demyelination were infiltrated by histiocytes and occasional round cells

Lesions in the spinal cord, meninges and ganglia were found at all levels, but most commonly in the lumbar segments

Cerebrum and brain stem Lesions in the brain were lacking in most of the experimental animals In the cases where they did occur only slight changes were observed such as occasional round cells in the subarachnoid space at the base of the brain, small subpial foci of destruction and microglia proliferation in the pons, infiltration of lymphocytes in the choroid plexus, small subependymal cell infiltrations and – in one case – a tiny peri- and paravascular collection of inflammatory cells in the white matter near one lateral ventricle

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In order to study true early lesions of EAF it seems most expedient to sacrifice the animals before the appearance of clinical manifestations

SUMMARY

Thirty-one rabbits injected with a mixture of bovine spinal cord and adjuvant were sacrificed early, *i e* before, or shortly after, the clinical onset of allergic encephalomyelitis The results of clinical and histological examinations and analyses of the cerebrospinal fluids are described and discussed in terms of pathogenesis

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FROM THE UNIVERSITY DEPARTMENT OF PATHOLOGY (HEAD PROFESSOR F LINELL) GENERAL HOSPITAL MALMÖ SWEDEN

THYMIC CYST OF THE NECK

Report of a Case

By

CH BEHRING and F BERGMAN

Received 20 ii 63

Various kinds of human thymic cysts are on record Thymic cyst of the neck is, however, rare The first case of a cystic tumour of the neck containing thymic tissue was reported by *Polloson & Piery* in 1901, since when 18 cases have been described (*Pezcoller* 1929, *Gilmour* 1937, *Hyde et al* 1944, *King* 1949 (8 cases), *Weller et al* 1951, *Lange Hellman* 1952, *Williams & Gerber* 1957, *Crawford et al* 1957, *Viar et al* 1959, *Cote & Fortin* 1961, *Gaeckle & Gerber* 1962)

A further case of this uncommon tumour is presented below

CASE REPORT

A previously healthy 9 year old boy was first seen at the E N T department because of an asymptomatic bulge in the neck The bulge was situated lateral to the left lobe of the thyroid gland and anterior to the sternocleidomastoid muscle

[illegible] course was uneventful

Gross findings The operative specimen was a smooth elongated tumour which measured 4 by 2 by 2.5 cm The cut surface showed a central unilocular cystic space containing a small amount of thick brown red liquid The lining of the cyst was smooth and the cyst wall was 0.5–1 cm thick and firm and its cut surface was grayish white The cyst wall contained a few microcysts filled with yellowish brown material

Microscopic findings The central large cyst was lined with [illegible] made up of cells which in some place[illegible]
Small papillary projections within th[illegible]
[illegible] epithelium In several areas the lin[illegible]

[illegible] Hassall's corpuscles
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Figs 3 and 4

Fig 3 Thymic tissue in wall of the cyst showing typical Hassall s corpuscles
Fig 4 Hassall s corpuscle with necrotic center

Figs 1 and 2

Fig 1 Lumen of the cyst with epithelial lining of varying thickness

Fig 2 Portion of the cyst lined by squamous epithelium



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Fig 1 Lumen of the cyst with epithelial lining of varying thickness

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lumen, which owing to inflammation of unknown cause, became cystic, dilated and clinically manifest However, cystic Hassall s corpuscles and sequestral cysts" were also present in the wall of the large cyst

A number of workers have demonstrated the simultaneous occurrence of thymus and parathyroid tissue in the neck The development of the parathyroid glands is intimately connected with that of the thymus The glands arise from the same pouch-complex, and during embryonal life the thymus primordium from the fourth branchial pouch (thymus IV) is situated together with the upper parathyroid gland beside the upper lobe of the thyroid gland In a study of developmental abnormalities of the thymus and parathyroids *Gilmour* (1937, 38, 39, 41) described *inter alia* a case of cystic thymus tissue in association with a parathyroid gland in the neck In our case a small island of parathyroid tissue was found adjacent to the wall of the thymic cyst and removed without any serious complications

Cystic thymus tissue of the neck may be located in any position along a line extending from the angle of the jaw medially to the midline of the neck All cases described were seen in infants and young persons The cysts, which may be unilocular or multilocular, appear to occur almost exclusively unilaterally They are often asymptomatic, but the mass might be painful if it becomes infected or if it grows rapidly If the cyst is located in the midline, it may cause dysphagia In the differential diagnosis branchial cleft cysts, cystic hygroma and, if the mass is in the midline, thyreoglossal duct cyst should be considered Roentgen examination is useful, particularly to show any mediastinal extension of the cervical cyst, i e information of value to the surgeon prior to surgical intervention In a case of cervical thymus gland described by *Arnheim & Gemson* (1950) the mass was situated partly in the mediastinum and operation was followed by pneumothorax

None of the reported cases of thymic cyst in the neck were malignant How[illegible] poter[illegible] into [illegible] 1954, *Pirkey* 1954) It therefore appears justified to extirpate all such tumours, whether cystic or not and even if they are asymptomatic and show no signs of malignancy

SUMMARY

Report of a unilateral thymic cyst with parathyroid tissue in the wall situated in the left side of the neck and probably arising from an embryonal remnant of the thymopharyngeal duct in a 9 year old boy

REFERENCES

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Fig 5
Parathyroid tissue in the outer surface of the tumour

some of the Hassall's corpuscles were necrotic, calcified or cystic (Fig 4) The wall also exhibited numerous irregular areas of necrotic tissue with cholesterol clefts and accumulations of foreign body giant cells, foam cells, lymphocytes and plasma cells Sections stained for fat (Scharlach R) showed that some of the giant cells contained fat droplets Some of the necrotic areas were lined with reticular cells resulting in the formation of a minute cyst The margin of the wall was made up of dense compressed fibrous connective tissue with a sparse admixture of lymphocytes The general appearance was that of a cyst within thymic tissue with signs of chronic inflammation There were no signs of malignancy In one section an islet of parathyroid tissue was seen in the outer surface of the tumour (Fig 5)

At after examination one year later no signs of recurrence or abnormalities were seen

DISCUSSION

Various theories of the pathogenesis of cysts in thymus gland tissue have been suggested Cysts of syphilitic origin are now extremely rare Persistent thymic tubular remnants or remnants of the branchial cleft epithelium in the thymus anlage are thought to undergo haemorrhage or to accumulate fluid with the formation of cysts as a result (*Ribbert* 1912) *King* (1949) rejects the assumption of any direct relationship between cysts of the neck and structures in the early embryo He called these neoplasms "lateral lympho-epithelial cysts" Regressive changes in Hassall's corpuscles and focal necrosis of medullary tissue producing "sequestral cysts" represent other varieties of cysts in thymus gland tissue (*Speer* 1938) We believe that the large cyst in our case probably arose within an aberrant thymus in the neck with pre existing tubular

lumen which owing to inflammation of unknown cause became cystic dilated and clinically manifest. However cystic Hassall's corpuscles and sequestral cysts were also present in the wall of the large cyst.

A number of workers have demonstrated the simultaneous occurrence of thymus and parathyroid tissue in the neck. The development of the parathyroid glands is intimately connected with that of the thymus. The glands arise from the same pouch complex and during embryonal life the thymus primordium from the fourth branchial pouch (thymus IV) is situated together with the upper parathyroid gland beside the upper lobe of the thyroid gland. In a study of developmental abnormalities of the thymus and parathyroids *Gilmour* (1937, 38, 39, 41) described *inter alia* a case of cystic thymus tissue in association with a parathyroid gland in the neck. In our case a small island of parathyroid tissue was found adjacent to the wall of the thymic cyst and removed without any serious complications.

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FROM THE UNIVERSITY OF BERGEN SCHOOL OF MEDICINE
THE GADE INSTITUTE DEPARTMENT OF PATHOLOGY BERGEN NORWAY
(HEAD PROFESSOR E WAALER MD)

THE SIGNIFICANCE OF THE PROTEIN CONTENT OF EHRLICH S ASCITES CARCINOMA

By

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The possible presence of a cytotoxic factor in untreated Ehrlich ascites carcinoma has been demonstrated both in vivo and in vitro (*Hartveit* 1962 and 1963 a) It has been shown that swelling and loss of cytoplasm (similar to the cellular response to immunological damage in a low protein medium) is followed by pyknosis and clumping of the tumour cells This pyknosis is not a more severe form of injury as the viability of the pyknotic cells is little reduced (*Hartveit* 1963 b) That is to say in early transplants there is evidence that the host defences are succeeding in injuring some of the tumour cells After a certain time however though there is still evidence that an immunological reaction is present—the cells are shrunken clumped together and joined by intercellular bridges—there is also evidence that this reaction is abortive as the tumour continues to grow in spite of it (*Hartveit* 1963 b)

The question then arises as to why this immunological reaction is abortive The reaction is a lytic one Thus it is possible that lytic inhibitors may be involved It is well known that the serum proteins may inhibit the lysis of erythrocytes in vitro and probably also in vivo (*Ponder* 1948) On this basis it could be expected that an increase in the protein content of the medium surrounding the Ehrlich ascites carcinoma cells in vivo might inhibit lysis of these cells also The finding that the number of injured tumour cells present increases with time in early transplants and is followed by a sudden change in the morphology of the tumour cells (which appear pyknotic) with no further evidence of lysis supports this idea as the prote[illegible]
ly[illegible]
of [illegible]
ly[illegible] content should not rise once pyknosis has occurred

The following experiments were designed to test this tentative hypothesis In experiment I the appearance of Ehrlich ascites carcinoma cells from both early (prepyknotic) and late (pyknotic) transplants

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The question then arises as to why this immunological reaction is abortive. The reaction is a lytic one. Thus it is possible that lytic inhibitors may be involved. It is well known that the serum proteins may inhibit the lysis of erythrocytes in vitro, and probably also in vivo (*Ponder* 1948). On this basis it could be expected that an increase in the protein content of the medium surrounding the Ehrlich ascites carcinoma cells *in vivo* might inhibit lysis of these cells also. The finding that the number of injured tumour cells present increases with time in early transplants and is followed by a sudden change in the morphology of the tumour cells (which appear pyknotic) with no further evidence of lysis supports this idea as the prote...

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was examined on phase contrast microscopy, both undiluted and in media of known protein concentration. As this experiment showed that tumour cells in a medium of low protein content could be recognized on phase contrast microscopy experiment II was set up to apply this finding to the tumour as it is in vivo. Experiment III was designed to see if the protein content of the medium rose after the tumour cells had become pyknotic.

MATERIAL AND METHODS

The mice used and the Ehrlich ascites carcinoma were similar to those used in previous experiments (*Hartveit* 1961), tumour from the 135th and 141st transplant generations being used.

TABLE 1

Ehrlich's Ascites Carcinoma—the Types of Tumour Cells in the different Transplants

Type of transplant	Prepyknotic	Early pyknotic	Pyknotic
Types of tumour cells	Normal and Type 1*	Pyknotic and Type 1*	Pyknotic and Type 2*

* See text

It has been shown previously (*Hartveit* 1963 c) that the type of tumour cell present in Ehrlich's ascites carcinoma varies with the time after transplantation. Thus it is possible to classify the transplants into three types as is shown in Table 1. Firstly there are the transplants in which pyknosis has not yet occurred; these

large tumour cells showing

there are the early pyknotic

a few type 1 cells. Then

thirdly, there are the pyknotic transplants which contain pyknotic tumour cells in the early stages. When the host is moribund these transplants also contain autolytic (type 2) cells. (See Figs. 1 and 2 in *Hartveit* 1963 c).

On examining preparations of Ehrlich's ascites carcinoma cells under phase contrast microscopy it must be remembered that the nucleus of the cell is large and that the cytoplasm is scanty. The light halo may also be disturbing—especially on photographs. The comparison to be made in the following experiments is between the narrow rim of cytoplasm and the medium in which the cells are suspended. This is illustrated diagrammatically in Fig. 1.

Experiment I

Five prepyknotic and 5 pyknotic tumours were obtained by collecting the tumour ascites from male and female mice that had each received an intraperitoneal injection of 0.1 ml of Ehrlich's ascites carcinoma (tumour cell count 1 180 000/mm³, tumour blood content—1 per cent) three or twelve days previously.

Wet preparations of the undiluted tumour were examined at once by phase contrast microscopy. Then one part of the tumour ascites was diluted with 10 parts of a solution of 1 g per cent bovine albumin in phosphate buffered physiological saline (a low protein medium). A further one part of the tumour ascites was diluted with 10 parts of a similar solution containing 2.4 g per cent bovine albumin (a high protein medium). Wet preparations of the cells in these media were examined at once by phase contrast microscopy.

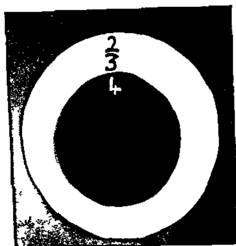


Fig 1

Diagrammatic representation of an Ehrlich ascites carcinoma cell on phase contrast microscopy
1 medium 2 light halo 3 cytoplasm 4 nucleus

Experiment II

One male mouse that had been given 0 1 ml of Ehrlich s ascites carcinoma 10 days before provided the tumour for the two experimental groups (A and B) each of which consisted of 5 male and 5 female mice Each of these was given 0 1 ml of the tumour ascites intraperitoneally (tumour cell count 1 290 000 mm^3 tumour blood content—2 per cent) The mice in group A were killed two days after transplantation and those in group B eight days after In all cases the tumour ascites was then removed films made and stained as described previously (*Hartveit* 1962), and wet preparations examined at once by phase contrast microscopy

Experiment III

One male mouse that had been given 0 1 ml of Ehrlich s ascites carcinoma intraperitoneally 10 days before provided the tumour for the two experimental groups (C and D) Group C consisted of 10 male and 10 female mice group D of 15 male and 1 [illegible] female m[illegible]

[illegible] were made from this and stained as in the previous experiment The tumour ascites was then centrifuged and the protein (plus nucleic acid) content of the cell free ascitic fluid estimated spectrophotometrically using a Unicam spectrophotometer and an extinction of 280 mμ

RESULTS

Table 2 gives a summary of the results

Experiment I

The findings are further illustrated in Figs 2 and 3 The cytoplasm of the tumour cells was dark in the undiluted prepyknotic tumours and in the same cells suspended in a medium of known low protein content

was examined on phase contrast microscopy, both undiluted and in media of known protein concentration As this experiment showed that tumour cells in a medium of low protein content could be recognized on phase contrast microscopy experiment II was set up to apply this finding to the tumour as it is in vivo Experiment III was designed to see if the protein content of the medium rose after the tumour cells had become pyknotic

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In all other cases it was bright Type 1 cells were only present when it was dark

Experiment II

Stained films Table 3 shows that the type of tumour varied in the two groups At two days most of the tumours were prepyknotic while at 8 days nine out of ten of the tumours had reached the pyknotic stage The sex differences in the findings are not statistically significant but the differences between the groups are (0 01 > P > 0 001) These results are in keeping with the results of serial biopsy reported earlier (*Hartveit* 1963 c)

TABLE 3

The Type of Tumour Present in Mice with Intraperitoneal Ehrlich s Ascites Carcinoma Related to Time after Transplantation (Leishman s Stain)

Group	Days after transplant	Type of tumour		
		Prepyknotic	Early pyk	Pyknotic
A	2	4♂ + 4♀	1♂	1♀
B	8	0	1♀	5♂ + 4♀

Phase contrast microscopy When these results were compared with those from the stained films it was found that in the cases where the tumours were at the prepyknotic stage (Fig 4 a) the cytoplasm of the cells was darker than the medium in the early pyknotic stage the cytoplasm was dark in some and light in other cells while in the pyknotic tumours (Fig 4 b) the cytoplasm was brighter than the medium in all cases (*Table 2*) Once again type 1 cells were only present when the cytoplasm was dark Thus in the light of the findings in experiment I the medium surrounding the tumour cells in prepyknotic transplants is probably of low protein content This is supported by *Ledoux & Revell*s (1955) findings with the Landschutz tumour in which the protein content of the ascitic fluid was lowest in early transplants

Experiment III

At eight days there were 8 male and 10 female survivors in group C and at twelve days 11 male and 8 female survivors in group D In group C the tumour cells had reached the pyknotic stage in all but three of the males As the experiment was designed to find the protein content of the pyknotic tumours these three animals were excluded from further investigations All the tumours in group D were pyknotic a few autolytic cells were present in some cases

Table 4 shows the mean protein content (g per cent) of these pyknotic tumours with its standard deviation (S D) The differences between the groups and between the sexes are not statistically significant

TABLE 2

Summary of Results

The Appearance of Ehrlich's Ascites Carcinoma Cells on Phase Contrast Microscopy in Media of different Protein Content
Low Protein Content—LPC High Protein Content—HPC

Experiment	Type of tumour	Medium	Cytoplasm	Type of tumour cell	Note
I	Prepyknotic	LPC	Dark	Normal + type 1	See Fig. 2 a
		HPC	Bright	Pyknotic	See Fig. 2 b
		Undiluted tumour	Dark	Normal + type 1	
	Pyknotic	LPC	Bright	Pyknotic	See Fig. 3 a
		HPC	Bright	Pyknotic	See Fig. 3 b
		Undiluted tumour	Bright	Pyknotic	
II	Prepyknotic	Undiluted tumour	Dark	Normal + type 1	
	Early pyk.	Undiluted tumour	Dark & bright	Pyknotic + type 1	
	Pyknotic	Undiluted tumour	Bright	Pyknotic	

Note: No sex differences were observed

a Cells suspended in low protein medium Note brightness of cytoplasm

b Cells suspended in high protein medium Note brightness of cytoplasm

Fig 3

Ehrlich ascites carcinoma cells *from a pyknotic* transplant suspended in media of different protein *concentration* (Phase contrast microscopy × 400)

a Cells suspended in low protein medium Note darkness of cytoplasm (→)

b Cells suspended in high protein medium Note brightness of cytoplasm (→)

Fig 2

Ehrlich ascites carcinoma cells from a *prepyknotic* transplant suspended in media of different protein concentration (Phase contrast microscopy × 400)

a Cells suspended in low protein medium Note brightness of cytoplasm

b Cells suspended in high protein medium Note brightness of cytoplasm

Fig 3

Ehrlich ascites carcinoma cells from a *pyknotic* transplant suspended in media of different protein concentration (Phase contrast microscopy × 400)

a A prepyknotic transplant Note darkness of cytoplasm (→) and particularly of cytoplasmic blebs

b A pyknotic transplant Note brightness of cytoplasm (→)

Fig 4

The appearance of Ehrlich ascites carcinoma cells on phase contrast microscopy (Undiluted specimen × 1100)

These results show that the protein content of the medium in these pyknotic tumours is high and that after the tumour has reached the pyknotic stage the protein content *of the medium does not increase* These findings are in keeping with those of *Kun Talalay & Williams Ashman* (1951) from pooled tumour ascites between 7 and 12 days after transplantation *Burgess & Sylven* (1962) give slightly higher values 3–4 g per cent but these were for a distinct subline of the tumour

TABLE 4

The Mean Protein Content ($\bar{x}$) plus S D of the Medium Surrounding Pyknotic Ehrlich Ascites Carcinoma Cells in vivo at 8 and 12 Days after Transplantation

Group	Days after transplant	Sexes	No of mice	$\bar{x}$ (g %)	S D $\bar{x}$
C	8	♂	5	2.76	0.65
		♀	10	2.57	0.89
D	12	♂	11	2.55	0.28
		♀	8	2.57	0.31

DISCUSSION

Experiment I *of the present paper shows that it is possible using* phase contrast microscopy to say whether an Ehrlich ascites carcinoma cell from a prepyknotic transplant is in a medium of high or low protein content as defined above The cytoplasm of the cells from these transplants appeared dark in media of low protein content and bright in media of high protein content On the other hand the cytoplasm of cells from pyknotic transplants appeared bright regardless of the protein content of the medium Thus while darkness of the cytoplasm can be taken as evidence that these cells are in a medium of low protein content the converse—that cells in which the cytoplasm is bright are in a high protein medium—is not necessarily true

Experiment II makes use of these findings to confirm that the medium in prepyknotic transplants is of low protein content as the cytoplasm was dark *in all cases The volume of these early* transplants is also low which makes direct estimation of the protein content difficult if pooled samples are to be avoided In addition other methods of estimating the protein content of the medium *involve centrifugation* to remove the tumour cells This process breaks up the type 1 cells present and renders subsequent estimations of the protein content of the medium meaningless (This objection to centrifugation of the samples does not hold in experiment III as type 1 cells were not present and pyknotic cells do not show morphological damage on centrifugation) The cytoplasm of the pyknotic cells was bright in all cases but no conclusions as *to the* protein content of the medium can be drawn from this finding However experiment III showed that the protein content

a A prepyknotic transplant Note darkness of cytoplasm (→), and particularly of cytoplasmic blebs

b A pyknotic transplant Note brightness of cytoplasm (→)

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The appearance of Ehrlich ascites carcinoma cells on phase contrast microscopy (Undiluted specimen × 1100)

pyknotic and pyknotic transplants appears bright in undiluted serum from normal mice, while if the serum is diluted 1 10 with physiological saline the prepyknotic and pyknotic cells both show dark cytoplasm The finding that the pyknotic cells also develop dark cytoplasm is of note, as this change is similar to that which occurs in vivo on transplantation As was shown in experiment I lowering of the protein content of the medium alone is not enough to bring about this change This suggests that mouse serum may contain a further factor that is needed to convert a pyknotic cell to a prepyknotic one In view of common experience with tumour specific antiserum in vitro it may be one of the components of complement that is needed If so this factor must be lacking in late transplants or the cytoplasm of the pyknotic cells would have become dark on reduction of the protein content of the medium Conversely, enough of this factor must be present in prepyknotic transplants to allow the immunological reaction to take place It is unlikely that lack of complement limits the reaction in vivo as the cytoplasm of the prepyknotic tumour cells diluted in a high protein medium appeared bright, while those diluted in a low protein medium appeared dark As the amount of complement would have been the same in both cases it cannot be this that determines the appearance of the cytoplasm, or the type of tumour cell present Thus the survival of the Ehrlich ascites carcinoma cells in the presence of the cytotoxic factor may well be due to the protective effect of the protein content of the ascitic fluid

SUMMARY

On the basis of the findings on phase contrast [illegible]

[illegible] content of the fluid in pyknotic transplants is high (about 2 5 g per cent) As cell damage of an immunological type is only seen in prepyknotic transplants the results of these [illegible]

[illegible] by the host in response to the homotransplanted tumour

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Amos [illegible] [illegible]nmune reactions [illegible] Basel/Stuttgart

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of the medium in pyknotic transplants is high and, in addition, that it does not increase with time

It is suggested that the increase in the protein content of the medium surrounding the cells that must take place if, as has been shown previously (*Hartveit* 1963 c), a prepyknotic tumour proceeds to become a pyknotic one, is the result of loss of protein from the type 1, the immunologically injured, cells It appears from previous results (*Hartveit* 1963 c) that when about 30 per cent of the tumour cells have lost their cytoplasmic protein to the medium the remaining tumour cells become pyknotic From that time on no further loss of protein to the medium takes place, as is shown in experiment III While only 30 per cent of the tumour cells show signs of gross injury all the remaining cells shrink, show clumping and intercellular bridge formation (*Hartveit* 1963 c). Thus all the cells show evidence of response to an immunological reaction although lysis does not result in all cases This is of note in view of *Flax's* (1956) and *Lindner's* (1960) reports that about 70 per cent of the tumour cells are insensitive to the effects of tumour specific antiserum

The above findings suggest that the high protein content of the medium surrounding the pyknotic tumour cells protects them from damage of an immunological type Green, Barrow & Goldberg (1959) have previously shown that such damage takes place in media of low protein content (about 0 5 g per cent), and this is in keeping with the findings in the present experiment But their findings in media of a "high" protein content, which have been confirmed by *Agol* (1961), cannot be compared with the findings in the present experiment as the "high" protein content is different In their work the high protein content was between 15 and 20 g per cent, a very unlikely situation physiologically They argue that swelling of the antibody treated tumour cells, in the presence of complement, was prevented osmotically in their high protein medium In the present experiments osmotic prevention of swelling cannot explain the results It seems more likely that the lytic reaction is inhibited by the proteins that are present in physiological amounts Here Amos's (1961) findings are of note, as he has shown that in some homografts (L 1210 in C3H mice), the tumour population becomes sensitized during the time of rejection and that sensitized cells tend to accumulate faster than they can be eliminated by phagocytosis or by lysis He goes on to state that "many of the cells are stained immediately by Trypan blue," (i e are of doubtful viability), "others after incubation in Ringer's solution" In other words more cell damage became evident when the protein content of the medium was reduced

It may be objected that the use of bovine albumin in the protein media used in the tests is artificial and that mouse protein might not act in the same way However, further control experiments have shown that the cytoplasm of Ehrlich's ascites carcinoma cells from both pre-

pyknotic and pyknotic transplants appears bright in undiluted serum from normal mice, while if the serum is diluted 1 10 with physiological saline the prepyknotic and pyknotic cells both show dark cytoplasm The finding that the pyknotic cells also develop dark cytoplasm is of note as this change is similar to that which occurs in vivo on transplantation As was shown in experiment I lowering of the protein content of the medium alone is not enough to bring about this change This suggests that mouse serum may contain a further factor that is needed to convert a pyknotic cell to a prepyknotic one In view of common experience with tumour specific antiserum in vitro it may be one of the components of complement that is needed If so this factor must be lacking in late transplants or the cytoplasm of the pyknotic cells would have become dark on reduction of the protein content of the medium Conversely, enough of this factor must be present in prepyknotic transplants to allow the immunological reaction to take place It is unlikely that lack of complement limits the reaction in vivo as the cytoplasm of the prepyknotic tumour cells diluted in a high protein medium appeared bright, while those diluted in a low protein medium appeared dark As the amount of complement would have been the same in both cases it cannot be this that determines the appearance of the cytoplasm, or the type of tumour cell present Thus the survival of the Ehrlich ascites carcinoma cells in the presence of the cytotoxic factor may well be due to the protective effect of the protein content of the ascitic fluid

SUMMARY

On the basis of the findings on [illegible]
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ascites carcinoma cells in vivo is [illegible]
protein content of the fluid in pyknotic transplants is high (about 2 5 g per cent) As cell damage of an immunological type is only seen in prepyknotic transplants the results of these experiments support the hypothesis that the proteins in the ascitic fluid may, by acting as a lytic inhibitor, protect the tumour cells from the action of the cytotoxic factor produced by the host in response to the homotransplanted tumour

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POLYNUCLEOTIDE PHOSPHORYLASE IN NEISSERIA MENINGITIDIS

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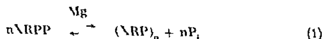
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The enzyme polynucleotide phosphorylase was first detected by *Grunberg-Manago & Ochoa* (1955) in the microorganism Azotobacter vinelandii. The enzyme was soon found to be present in a number of gram positive as well as gram negative bacteria. It is found in aerobic as well as in anaerobic species (*Brummond, Staehelin & Ochoa* 1947, *Littauer & Kornberg* 1957, *Beers* 1957). In some organisms, however, such an enzyme activity has not been demonstrated. Such species are the gram positive Lactobacillus arabinosus and Clostridium acetobutyricum (*Brummond, Staehelin & Ochoa* 1957). In many other clostridia the polynucleotide phosphorylase is found in large quantities. The enzyme has not yet been demonstrated unequivocally in animal cells. Apparently, however, it is present in liver nuclei and in Ascaris lumbricoides (*Hilmoe & Heppel* 1957, *Entner & Gonzalez* 1959).

The properties of polynucleotide phosphorylase seem to differ in various bacterial species. The enzymes from Escherichia coli and Azotobacter vinelandii have similar properties, whereas those from Micrococcus lysodeikticus and Clostridium perfringens seem to be somewhat different (*Grunberg-Manago, Ortiz & Ochoa* 1956, *Littauer & Kornberg* 1957, *Beers* 1957, *Dolin* 1961, *Grunberg-Manago* 1962).

Polynucleotide phosphorylase is usually found in the soluble portion of the cell, although ribosomes of E. coli and Pseudomonas aeruginosa contained some activity (*Wade & Lovett* 1961, *Strasdine, Hogg & Campbell* 1962). In Streptococcus faecalis, however, the enzyme is found in cell membranes (*Abrams & McNamara* 1962).

Polynucleotide phosphorylase catalyses the synthesis of long chain polynucleotides from nucleoside 5'-diphosphates with elimination of inorganic phosphate (*Grunberg-Manago, Ortiz & Ochoa* 1956).



$$nXRPP \overset{Mg}{\rightleftharpoons} (XRP)_n + nP_i \qquad (1)$$

In this equation X is a purine or pyrimidin base, or a mixture of bases, R is ribose and P phosphate.

There are probably four reactions catalysed by the enzyme a) A synthesis according to equation 1 b) A phosphorolytic cleavage of polyribonucleotides according to a reverse equation 1 c) An exchange between $32P_i$ and the terminal phosphate of the nucleoside diphosphate d) A transfer of nucleoside diphosphate units from a polynucleotide donor to a polynucleotide acceptor (*Singer, Heppel, Hilmoe, Ochoa & Mii* 1959) The first three reactions have been used in the assay of the enzyme

The starting point of the present investigation was the observation that ADP upon addition to respiring Neisseria meningitidis suspensions or extracts, became radioactive when $32P_i$ is present When ADP had become labelled it also conferred radioactivity to other cell constituents Such radioactivity incorporation resulted in high blank values in studies in which oxidative phosphorylation was the subject Accordingly, investigations were started to determine the various ways in which $32P_i$ may be assimilated by exchange in the meningococcal system Only when these ways are known, a study of the oxidative phosphorylation may be safely undertaken

MATERIALS AND METHODS

Meningococcal strains The meningococcal strains were the same which have previously been used The test microbe was generally the meningococcus strain M6 adapted to growth on a minimal medium (*Jyssum & Jyssum* 1962 a) The methods used for growth and harvesting have been described previously

Cell free extracts An extraction after ultrasonic disintegration of the cells was used as the regular procedure (*Jyssum & Jyssum* 1962 a) The crude extracts which contained approximately 4 mg N_2 per ml were diluted 1 to 4 either with 0 1 M tris buffer pH 8 or with 0 1 M glycine NaOH buffer pH 9 and dialysed for 1 h at 4° against redistilled water with external and internal stirring

Analytical procedures In the absence of inorganic phosphate the enzyme was assayed by measurement of the release of inorganic phosphate upon the addition of ADP The ADP solution was made from stock solutions brought to pH 8 with KOH Inorganic phosphate was determined by the method of Fiske & Subbarow such as previously used (*Jyssum & Jyssum* 1962 a) The P_i liberated during the course of an incubation of substrates with a cell free fraction was always corrected for P_i in control incubation mixtures without added substrate or cell fraction The incubations were carried out at 37° C

The enzyme activity was also assayed by determining the incorporation of radio active ortophosphate This procedure was based upon that used by *Grunberg Manago Ortiz & Ochoa* (1956) For the determination of a total uptake of ortophos

' *Nielsen & Lehninger* (1955)
ter Zetterstrom & Lindberg
it of *Martin & Doty* (1949)

The samples were counted wet in aluminium planchets under constant geometry From the specific activity of the ortophosphate present in the reaction medium the radioactivity of the aliquots of incorporated P_i and appropriate factors for aliquot size dilution etc the amount of ortophosphate uptake per ml of the original reaction mixture was calculated

The exchange of $32P_i$ into nucleoside phosphates was determined after isolation of the latter by chromatography according to the method of *Krebs & Hems* (1953) The positions of the nucleotides on the paper were located with ultraviolet light and marked in pencil The radioactivity was located by strip chart recording The relative activities of the spots were determined by planimetry and calculated in

term of P_i by means of spots from the ortophosphate originally added to the reaction medium. Such spots were included in each scanning.

The polymerization was also followed by measurements of the polyribonucleotides synthetized during the course of an experiment (*Grunberg-Manago, Ortiz & Ochoa* 1956). After precipitation of the reaction mixture either with ice cold 30 per cent TCA or 5 per cent $HClO_4$ the sediments were washed with 3 ml of the acid used and then twice with ice cold 95 per cent ethanol. The sediment was then dried overnight at 37° (*Warner* 1957). The dried powder was dissolved in 0.1 M tris at pH 8. Aliquots were used for a determination of $^{32}P_i$ incorporation and for a scanning between the wavelengths 290 mμ and 220 mμ. The polymerization products were also further examined after dialysis for 24 h against water followed by lyophilization.

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fuged for 1½ h. Next the pellet was resuspended and centrifuged for another 1 h. The resulting pellet was resuspended and used as the "particle" fraction. The supernatant from the first centrifugation was also run for another 1 h before use.

Equipment. Spectrophotometry was performed either with the Hilger and Watts Uvispec spectrophotometer or in a Beckman model DB spectrophotometer with potentiometric strip chart recorder. In both instruments analyses were performed with 1 cm light path.

Radioactivity was measured with a Frieseke and Hoepfner gass flow counter equipped with a thin window. The activity distribution in paper chromatograms was recorded by means of paper strip recording in an automatic paper chromatograph. *Planimetry* of the diagrams was performed with an "Aristo" planimeter model 1100

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RESULTS

Reaction of ADP in the presence of meningococcal extract. The liberation of P_i from ADP incubated with meningococcus extract is illustrated in Fig. 1 and Fig. 2. The clearing of P_i from ADP by meningococcal extracts was accompanied by a progressive hypochromicity of the supernatant obtained after precipitation with TCA or $HClO_4$ (*Warner* 1957). This feature which is illustrated by the absorbancy at 260 mμ included in Fig. 2 is in agreement with the activity of a polynucleotide phosphorylase.

The rate of liberation of P_i is also dependent on the enzyme concentration as well as of the concentration of ADP. The relation between the P_i recovered and the ADP concentration appears from some data presented in Fig. 1.

The activity of the polynucleotide phosphorylase is known to be a function of the Mg^{++} concentration (*Grunberg-Manago & Ochoa* 1955, *Beers* 1957). From the data of Fig. 3 it is seen that phosphate liberation as well as the hypochromicity developed in the meningococcal system

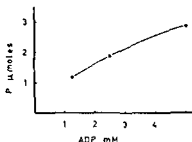


Fig 1

Liberation of P_i from various concentrations of ADP by extracts from N meningitidis

A total volume of 2 ml contained 150 µmoles tris as buffer pH 7 5 2 µmoles $MgCl_2$ and 0 2 ml dialysed meningococcus extract dilution Incubated at 37° for 30 minutes and stopped by the addition of 2 ml 5 per cent $HClO_4$ Analysis performed on the supernatant fluid

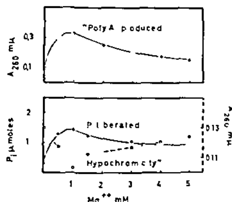


Fig 3

Variations in the activity of the polynucleotide phosphorylase of N meningitidis with the Mg^{++} concentration

The system was analogous to that described in the text to Fig 2 The reaction was run at 37° for 30 minutes

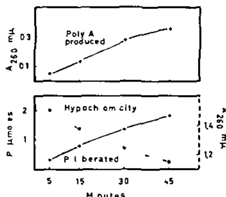


Fig 2

Variations in the activity of the polynucleotide phosphorylase of N meningitidis with time

A total volume of 2 ml contained 165 µmoles tris as buffer pH 8 2 µmoles $MgSO_4$ 5 µmoles ADP and 0 2 ml meningococcus extract dilution The reaction was run at 37° and stopped by the addition of 2 ml 4 per cent $HClO_4$ Supernatants were analysed for P_i and for the development of hypochromicity Sediments analysed for Poly A Endogenous values substracted

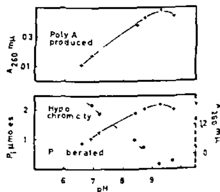


Fig 4

Variations in the activity of the polynucleotide phosphorylase of N meningitidis with pH

The system was analogous to that described in the text to Fig 2 but with either tris HCl or glycine-NaOH buffer at the desired pH The reaction was run for 30 minutes at 37°

is also strongly influenced by the Mg^{++} concentration In the experimental series presented the rate of the reaction appears to increase as a function of the Mg^{++} concentration until an optimal concentration, approximately 1 mM is reached *Further experiments seem to indicate* that Mg^{++} is essential and cannot be replaced by other divalent cations

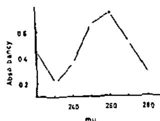


Fig 5
The ultraviolet absorption spectrum of the polymer synthetized by meningococcal extracts from ADP

Mn^{++} could not catalyse the reaction, and was even inhibitory in the presence of Mg^{++} (*Littauer & Kornberg* 1957)

In accordance with the properties of enzymes from other bacteria the activity in meningococcal extracts was found to be optimal at a high pH (*Grunberg-Manago & Ochoa* 1955, *Beers* 1957) Some data which illustrate the effect of pH have been recorded in Fig 4 A further analysis of the range between pH 8 and pH 10 seems to indicate a maximum for the activity between pH 8 7 and pH 9 3 at the Mg^{++} concentration previously found optimal

Synthesis of polymer The polymer obtained from ADP ("Poly A") is precipitated by $HClO_4$, and can be recovered from the precipitate in the way it has been described under methods In Fig 5 the ultraviolet absorption spectrum of the polymer synthetized by meningococcal extracts from ADP has been presented When measurements at 260 mμ are used as the analytical procedure, a quantitative assay may be arranged for the synthesis of the polymer (*Warner* 1957) The synthesis of polyribonucleotides in the previously described experiments was followed in this technique The data have been included in the figures 2 3 and 4 It is seen that the quantity of polymer synthetized follows the quantity of P_i released as well as the degree of hypochromicity found in the supernatant fluid

Nucleoside phosphate—32P$_i$ exchange One of the characteristic reactions catalysed by nucleotide phosphorylase of other organisms is the ADP—32P$_i$ exchange (*Grunberg-Manago & Ochoa* 1955) Such an exchange reaction was also catalysed by the meningococcal extracts When ADP was incubated with the cell free extracts in the presence of 32P$_i$ the ADP became labelled such as shown in the experiments presented in Table 1 The formation of labelled ATP in these experiments indicates the presence of an adenylate kinase in the meningococcal extracts



$$AMP + ATP \rightleftharpoons 2\,ADP \qquad (2)$$

TABLE 1

$^{32}P_i$ Exchange with ADP and ATP in the Presence of Meningococcal Extract

Nucleoside phosphate added (5 µmoles)	$^{32}P_i$ incorporated in nucleoside phosphates (mµmoles)		
	AMP	ADP	ATP
AMP	–	–	–
ADP	–	137	159
ATP	–	–	16
AMP + ATP	–	61	62

The system contained in a total volume of 2 ml 145 µmoles glycine NaOH buffer pH 9, 2 µmoles $MgSO_4$ 6 4 µmoles $^{32}P_i$ as ortophosphate Experiments were run for 30 minutes at 37° and stopped with 0 2 ml 30 per cent TCA Analysed by chromatography, strip chart recording and planimetry as described under methods

TABLE 2

Variation in the Exchange between $^{32}P_i$ and ADP with the ADP Concentration

ADP added (µmoles)	$^{32}P_i$ incorporated (mµmoles)	ADP $^{32}P_i$
–	2 6	–
2 5	231 2	0 39
5 0	739 4	0 78
7 5	533 0	1 17
10 0	371 2	1 56
15 0	237 6	2 34

A total volume of 2 ml contained 145 µmoles glycine—NaOH buffer pH 9 2 $MgSO_4$ 2 µmoles, 0 2 ml dialysed meningococcus extract dilution After 30 minutes incubation the reaction was stopped with 0 2 ml 30 per cent TCA and analysed as described under methods

The absence of $^{32}P_i$ in AMP is in agreement with such an enzyme reaction A faint labelling of ATP alone may be the result of a coupling of the adenylate kinase with an ATP-ase reaction (*Jyssum & Jyssum* 1962 b) Catalytic amounts of ADP may also be thought to arise by phosphorolysis of polynucleotides present in the crude extracts by the reversed reaction corresponding to equation 1

Maximal activity in the exchange assay required approximately 5 0 mM ADP, giving a ratio 0 78 between ADP and $^{32}P_i$ In accordance with the findings of *Grunberg-Manago, Ortiz & Ochoa* (1956) the reaction was found to decrease rather sharply for values of the ratio above or below this optimum This effect has been illustrated by the experiment recorded in Table 2

Substrate specificity In a following series of analyses the relative rate of incorporation of $^{32}P_i$ into other nucleoside phosphates was studied In Table 3 some data obtained in these experiments have been recorded

Radioactive ortophosphate is readily incorporated in the nucleoside

TABLE 3

$32P_i$ exchange with Various Nucleoside Phosphates in the Presence of Meningococcal Extract

Nucleoside phosphate added (2.5 μmoles)	Total incorporation of $32P_i$ Wet count (mμmoles)	Incorporated into polynucleotides (mμmoles)	$32P_i$ incorporated into the nucleoside phosphates (mμmoles)						
			ADP	ATP	CDP	GDP	IDP	ITP	UDP
ADP	607	3 6	45	67					
CDP	1003	13 9			201				
GDP	682	2 1				222			
IDP	878	3 5					214		
ITP*	26	9 5						trace	
UPD	499	7 7							106
–	trace	trace							

The experimental system was analogous to that described in the text to Table 1
* Spot also corresponding to IDP in the chromatogram

diphosphates tested In contrast to this, ATP is only significantly labelled from ADP, a feature which seems to emphasize the substrate specificity of the adenylate kinase in Neisseria meningitidis

It should be pointed out that no significant uptake of $32P_i$ is obtained without added nucleoside diphosphate One important feature of the present experiments is the large amount of $32P_i$ which is incorporated into other phosphorus containing compounds ("esterified phosphate") in the system than the nucleoside phosphates and the polynucleotides actually measured These compounds are moving with the ortophosphate and the pyrophosphate in the HCOOH—isopropylether solvent of Hanes and Isherwood (*Krebs & Hems* 1953) The nature of these substances has not been considered a subject of the present investigation

From the data presented in Table 3 it is seen that the exchange reactions were followed by the measurement of a synthesis of substances with general properties of the polyribonucleotides In accordance with the recognized properties of the polynucleotide phosphorylase these polymers contained only *trace* amounts of radioactivity

Localization of the activity In order to find out whether the phosphorylase activity in Neisseria meningitidis is a soluble system the crude extract was separated into supernatant and "particles" in the ultracentrifuge such as described under methods When the resulting fractions were examined by the total incorporation of P_i in the exchange technique, data like those recorded in Table 4 were obtained The experiments are taken to indicate that the enzyme is residing in the soluble fraction No significant activity can be demonstrated in the "particle" fraction

TABLE 4

Localization of the Polynucleotide Phosphorylase Activity in Crude Meningococcal Extract

Fraction examined	Corresponding volume of crude extract	$^{32}P_i$ incorporated (mµmoles)
Crude extract	0 05 ml	237 2
Supernatant	0 05 ml	222 9
'Particles"	0 05 ml	3 7
'Particles' + Supernatant	0 05 ml	234 2

The system contained in a total volume of 2ml 80 µmoles tris as buffer pH 6 8 EDTA 2 µmoles KCN 1 µmole, $MgSO_4$ 10 µmoles ADP 5 µmoles and 32 P_i 6 4 µmoles The exchange was determined as total incorporation by "wet count' as described under methods

DISCUSSION

The data which have been presented show that dialysed cell free extracts from Neisseria meningitidis catalyse a net synthesis of polyribonucleotides from ADP under the release of inorganic phosphate The rate of synthesis appears to be proportional to the initial concentration of ADP as well as of the enzyme concentration The rate depends on the concentration of Mg^{++} At low concentrations the reaction increases with the cation concentration until an optimum is reached at approximately 1 mM At higher concentrations the rate of the reaction appears to fall off, approaching a minimum In the meningococcal system Mg^{++} seems to be essential and cannot be replaced by other divalent cations Unlike the system described in Micrococcus lysodeikticus (*Beers* 1957) the meningococcal enzyme is inhibited by Mn^{++} in the presence of Mg^{++} This is more like the properties of the enzyme in Escherichia coli (*Littauer & Kornberg* 1956) The activity of the enzyme increases with increasing pH until an optimum is reached between pH 8 7 and pH 9 3 This appears more like the findings from studies of Micrococcus lysodeikticus (*Beers* 1957) than those from studies of Azotobacter vinelandii since the enzyme of the latter microbe apparently has its optimum at pH 8 1 (*Grunberg-Manago, Ortiz & Ochoa* 1956) The soluble, non dialysable polymeric product of the reaction exhibited a typical nucleotide spectrum in the ultraviolet region The quantity synthetized of this polymer coincided with the disappearance of substrate ADP as well as the appearance of inorganic phosphate with regard to changes in enzyme concentration time of incubation, ADP concentration Mg^{++} concentration and H ion concentration

An ADP—32P_i exchange reaction had the characteristics of a polynucleotide phosphorylase such as isolated from other organisms The 32P_i exchange assays also demonstrate the presence of an adenylate kinase in the extracts under study The latter enzyme activity may be inhibited by F according to the data of *Grunberg Manago, Ortiz & Ochoa*

(1956) with very little effect on the phosphorylase reaction Radioactive ortophosphate was also readily incorporated into other nucleoside diphosphates in the way it is reported in Table 3 Since the $32P_i$—ADP exchange is not always indicative of polynucleotide phosphorylase activity (*Grunberg-Manago* 1962) these exchange data are not unequivocally indicative of this enzyme in Neisseria meningitidis In each instance, however, the reaction was also followed by a simultaneous demonstration of the synthesis of substances with the general properties of polyribonucleotide complexes Thus, the present exchange reactions are taken to demonstrate a phosphorylase activity

The experiments with $32P_i$ showed that large amounts of labelled phosphate were incorporated into other "esterified phosphorus compounds' than the nucleoside phosphates and the polyribonucleotides Since this incorporation depends on the presence of one of the nucleoside diphosphates, it may be assumed that the latter substances exchange their terminal phosphorus groups with one or several substances already present Another explanation would be that the nucleoside diphosphates are incorporated into macromolecules in such a way that both phosphorus groups are included

According to the experiments performed no evidence is available that an oligonucleotide primer is essential for polymer formation in the meningococcal system It is likely, however, that trace amounts of such primers are present as contaminants in the crude enzyme preparations under study (*Singer, Heppel & Hilmoe* 1957) In other systems GDP polymerization shows a very slow rate compared with that of other nucleoside diphosphates (*Littauer & Kornberg* 1956) It is remarkable that a *net synthesis of* polyribonucleotides could be obtained with the crude meningococcal extracts used This seems to be in contrast to the experiments of *Brummond, Staehelin & Ochoa* (1957) with several other bacterial species, and may signify a low content or absence of nucleases in the meningococcal extracts

The polynucleotide phosphorylase of crude meningococcal extracts remained active after storage at —18° for more than two months

The phosphorylase activity is apparently residing in the soluble portion of the crude extract "Particles' obtained after centrifugation at approximately 225 000 g were without significant activity

SUMMARY

Cell free extracts from Neisseria meningitidis contain a polynucleotide phosphorylase Polynucleotides are produced from ADP, CPD, GDP, IDP and UDP respectively

The extracts catalyse an exchange between nucleoside diphosphates and radioactivity labelled ortophosphate The diphosphates thus labelled also confer radioactivity to other compounds in the dialysed meningococcal extracts

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An ADP—32P_i exchange reaction had the characteristics of a polynucleotide phosphorylase such as isolated from other organisms The 32P_i exchange assays also demonstrate the presence of an adenylate kinase in the extracts under study The latter enzyme activity may be inhibited by F^- according to the data of *Grunberg Manago, Ortiz & Ochoa*

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COMPARISON OF THE AGGLUTINABILITY BY RHEUMATOID ARTHRITIC SERA OF SENSITIZED SHEEP CELLS AND OF SENSITIZED SHEEP CELLS, EXPOSED TO HUMAN C'1, C'4 AND C'2

By

ANNA BRITA LAURELL

Received 22 ii 63

A characteristic property of complement is its ability to react with antigen antibody complexes Also the rheumatoid arthritic factor (RAF) can combine with some antigen-antibody complexes (*Waaler 1940*, *Fox et al* 1954, *Fox & Ballata* 1956, *Grubb* 1956, 1961, *Milgrom et al* 1956 *Waller & Vaughan* 1956) In an investigation of rheumatoid arthritic sera no correlation was found between the titres of complement or complement factors and of RAF (*Laurell & Grubb* 1958)

In the present investigation the agglutinability of sensitized sheep cells (EA) was compared with that of sensitized sheep cells complexed with the first, fourth and second components of human complement (*EA_{142}-cells*) The agglutination of EA_{142} cells by RAF was strongly suppressed compared with that of FA cells The study also showed that cells carrying (EA$_4$) exhibited the ule the agglutinability ... complement (EA_1 cells)

However, on addition of a goat anti rabbit globulin serum EA and EA_{142} cells agglutinated to the same degree

MATERIAL AND METHODS

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Normal pooled human serum was used as source of *complement* The pool was stored at −50° C

R1 R2 and R4 reagents for determining C'1, C'2 and C'4 activities were prepared

This investigation was supported by a *grant* from *Alfred Österlund s Foundation*

Meningococcal extracts contain an adenylic kinase which results in labelling of ATP from ADP

The properties of the polynucleotide phosphorylase have been examined with regard to substrate specificity, cation cofactor requirements and pH optimum

The polynucleotide phosphorylase activity is residing in the soluble fraction of meningococcal extracts Particles obtained after centrifugation at 225 000 g are without significant activity

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S nce the EA_{14} cells were prepared by exposing EA cells to human R3 conta ning C 1 C 2 and C 4 experiments were also performed with EA cells exposed to different preparat ons of human R3 that had been heated at 56° C for 30 minutes The rheumatoid arthritic sera agglu tinated such cells to the same titre as EA cells

After incubation of rheumatoid arthritic serum with EA_{142} cells the cells were centrifuged off and the agglutinating effect of the super natant on EA cells was assessed No inactivation of the rheumatoid arthritic factor was found

FA_{142} cells which had been incubated for 16–18 hours with rheuma toid arthritic serum were washed twice in veronal buffer and the cells were resuspended in veronal buffer Human complement chelated human complement and R4 respectively were added to these cells and the percentage haemolysis was read after 30 minutes at 37° C

The cells originally in the FA_{14} state underwent complete lysis when incubated with human complement and 50 per cent lysis on in cubation with R4 No lysis occurred on incubation with chelated com plement indicating that the cells has decayed to the EA_{14} state during incubation

Agglutinating Effect of Rheumatoid Arthritic Serum on EA Cells and Various Complexes of EA Cells with C 1 C 2 and C 4

Table 2 shows the agglutination of EA EA_1 EA_{14} EA_{14} EA_4 and FA_4 cells by one of the rheumatoid arthritic sera

No difference was observed between the agglutinability of EA and FA_1 cells by rheumatoid arthritic sera

All cell suspensions exerting C 4 activity were less agglutinable by rheumatoid arthritic sera than the EA cells No difference in agglutina bility could be shown betwen EA_{14} and EA_{14} cells or LA_{42} cells and FA_4 cells indicating that haemolytically active C 2 on the cells was not responsible for the decreased agglutinability

TABLF 2

Agglut nat on by Rheumat c Arthr t c Serum of EA and of EA Complexed w th Various Components of Human Complement

	FA	FA	FA e	FA e (3h s0° C)	EA e	EA	EA
RAS 1 5	4	4	3	3	3	4	3
10	4	4	3	3	3	3	3
20	4	4	3	3	3	3	3
40	4	4	3	3	3	2	2
80	4	4	1	2	1	1	1
160	4	4	1	1	1	0	1
320	4	3	1	1	0	0	0
640	2	2	0	1	0	0	0
1280	2	2	0	1	0	0	0
Control saline	0	0	0	1	0	0	0

according to *Kabat* & *Mayer* (1961). R4 was prepared by incubation of complement with hydrazin.

Human R3 was prepared according to *Leon* (1958) by incubation of pooled human serum with zymosan (Fleishmann's zymosan lot 5 B 171) at 37° C for 60 minutes after which the zymosan was centrifuged off. This R3 reagent was used for preparing FA_{142} cells.

Sensitized sheep erythrocytes (EA) were prepared according to *Pillemer et al.* (1956). Rabbit anti sheep red cell serum was used as amboceptor.

EA_{142} cells was prepared according to *Müller-Eberhard & Nilsson* (1960) with the exception that human R3 prepared as described above instead of guinea pig R3 was used as the source of C 1, C 2 and C 4. After having been incubated and then washed in the cold the EA_{142} cells were suspended in veronal buffer. The concentration of these cells was the same as that in the EA suspension (2.5×10^8 cells per ml). The EA_{142} cells showed 80–100 per cent lysis after incubation at 37° C for 30 minutes with excess of human complement in 0.01 M EDTA; when tested with an R4 preparation 100 per cent lysis was noted. No lysis was observed after incubation of the cells with 0.01 M EDTA in veronal buffer at 37° C for 30 minutes. The cell suspension was kept at 0° C and tests were performed within 4–6 hours.

EA_{14} cells were prepared by incubating the EA_{142} cells at 37° C for 60 minutes. The EA_{14} cells did not lyse on incubation with EDTA serum but they did on addition of R4 or R1.

EA_1 cells were prepared according to *Laporte et al.* (1957) by exposing the sensitized cells to the euglobulin fraction of human R4.

EA_{12} cells were prepared according to *Becker* (1960) by incubating EA_{14} cells in buffer containing 5×10^{-3} M EDTA for 3 hours at 0° C after which the cells were washed in the cold and resuspended in buffer.

EA_4 cells were obtained by incubating the EA_{42} cells at 37° C for 90 minutes.

The various cell suspensions were examined for C 1, C 2 and C 4 activities by human R1, R2 and R4 reagents.

Agglutination test. To 0.25 ml of each of the various erythrocyte suspensions was added 0.25 ml of serial dilutions of rheumatoid arthritic sera in veronal buffer. The degree of agglutination was noted after 16–18 hours at 20° C.

Goat Anti rabbit Globulin (Microbiological Associates Inc. Bethesda, USA).

RESULTS

The agglutinability of EA and EA_{142} cells by 10 rheumatoid arthritic sera was investigated. The agglutination of the EA_{14} cells compared with that of the EA cells was decreased by all 10 sera. The results with 3 of the sera are given in Table 1.

TABLE 1

Agglutination of EA and EA_{142} Cells by Rheumatoid Arthritic Sera

Serum dilution	RAS 30/58		RAS 43/58		RAS 8/58	
	EA	EA_{14}	EA	EA_{14}	EA	EA_{14}
1/20	4	2	4	1	4	0
40	4	1	4	1	4	0
80	4	1	4	0	4	0
160	4	1	4	0	3	0
320	4	1	4	0	2	0
640	2	1	4	0	1	0
1280	2	0	2	0	0	0
2560	1	0	2	0	0	0
control saline	0	0	0	0	0	0

0–4 = strength of agglutination. O = No agglutination.
4 = strong agglutination.

rheumatoid arthritic serum on sensitized sheep red cells and on γ globulin coated latex particles the findings in the present work support the view of Brine et al

Judging from the observations set forth above the decreased agglutinability of EA_{142} cells may be ascribable to the effect of C 4. The inagglutinability of the sensitized cells was independent of fixation of C 1 to the cells and of the presence of the haemolytic activity of C 2 on the cells

The most striking finding was the decreased agglutinability of cells possessing C 4 activity which might depend on a steric hindrance by C 4 on the cells or a destructive effect of C 4 on the structure of the γ globulin combining with RAF

Further the findings argued for the assumption that the group located on the rabbit amboceptor and reacting with RAF are different from those reacting with antibodies prepared by immunization with rabbit globulin

SUMMARY

1 Rheumatoid arthritic sera agglutinated sensitized sheep erythrocytes complexed with human C 1, C 4 and C 2 (EA_{142}) less than sensitized sheep erythrocytes (EA)

2 The decreased agglutinability was related to C 4 activity on the sensitized cells C 1 and haemolytically active C 2 had no agglutination inhibiting effect

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TABLE 3

Agglutination of FA and EA_{142} Cells by Anti Rabbit Gamma Globulin Serum

	EA	EA_{142}
Antirabbit gamma globulin 1/5	4	4
1/10	4	4
1/20	4	4
1/40	4	4
1/80	3	3
1/160	2	1
1/320	1	0
1/640	0	0

0–4 = strength of agglutination

Agglutination of EA and EA_{142} Cells by Goat Anti Rabbit Globulin Serum

Table 3 shows the agglutination of EA_{142} and EA cells by a goat anti rabbit globulin serum

No difference was found between the agglutinability of the two cell suspensions The same results were obtained when the amboceptor was varied from 4 to 12 times the minimal sensitizing dose

DISCUSSION

EA_{142} cells were less agglutinable by rheumatoid arthritic serum than EA cells (Table 1) Since EA_{142} were prepared by exposing EA cells to human R3, also serum components other than C'1, C'4 and C'2 might be adsorbed to the EA cells and inhibit the agglutination However, EA cells exposed to heated R3 did not show any decreased agglutinability by rheumatoid arthritic serum This favours the view that interaction of the complement components with the EA cells was responsible for the decreased agglutinability by rheumatoid arthritic factor (RAF)

Evidence obtained indicates that RAF was not destroyed or bound by the EA_{142} cells

Brine et al (1958) demonstrated the presence of a prozone in the latex fixation test when fresh rheumatoid sera were tested This inhibition in high concentrations of fresh rheumatoid sera was abolished on heating sera at 56° C for 30 minutes By testing serum reagents lacking individual components of complement they showed that the prozone could be explained by an interaction of the gamma-globulin coated latex particles with C'4 and C'2

In contrast, *Schubart* (1959) considered his results to warrant the conclusion that the prozone appearing in the latex fixation test when fresh rheumatoid sera were used, was probably due to the first component of complement

Assuming an analogy between the agglutination promoting effect of

FROM BACTERIOLOGICAL INSTITUTE UNIVERSITY OF LUND SWEDEN (HEAD PROFESSOR R GRUBB) AND FROM DEPARTMENT OF CLINICAL CHEMISTRY MALMÖ GENERAL HOSPITAL UNIVERSITY OF LUND SWEDEN (HEAD C B LAURELL MD)

ANTICOMPLEMENTARY EFFECT OF M COMPONENTS

By

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Received 22 ii 63

Sera from patients with myeloma may become anticomplementary, sometimes markedly so, on exposure to heat (*Jersild* 1939, *Olhagen* 1945 *Norgaard* 1955) This also holds for sera from cases with hyper gammaglobulinaemia of the type found in rheumatoid arthritis dis seminatus lupus and liver diseases It has recently been shown that the anticomplementary activity of normal γ globulin induced by heating or by adsorption to bentonite could be ascribed to an inactivation of the first fourth and second complement factors by γ globulin aggregates (*Ishizaka & Ishizaka* 1959 *Christian* 1961, *Marcus* 1961) Native γ globulin was much less anticomplementary

In this paper the anticomplementary effect of M components isolated from myeloma sera is described and compared with that of purified normal γ-globulin

MATERIAL

Purified M-components from sera from 9 patients with myeloma were studied The preparative procedure and the Gm typing system is described elsewhere (*Nils son* Three of the M components (196 611 and 619) were isolated by combined precipitation with ammonium sulphate and chromatography on DEAE cellulose Four (424 610 612 and 627) were isolated through fractionated precipitation with Rivanol and ammon um sulphate and one (559) by Rivanol and salt and further purification by preparative [illegible]

[illegible] gamma type (γ 7Sγ γss)

The electrophoretic homogeneity and content of normal gammaglobulin of the M-component solutions was stud ed by paper [illegible]
and by h rizontal st [illegible]
196 559 567 610 61 [illegible]
ponent 619 gave rise [illegible]

This investigati n was supported by grants from *Swedish Medical Research Council* and from *Alfred Österlunds Foundation*

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than normal γ globulin and did not increase their effect on heating (Table 1)

TABLE 1

Anticomplementary Activity of Heated M Components and Normal "γ" Globulin in Relation to Aggregation capacity Gm Characters Electrophoretic Mobility and Grade of Purity

	Lowest amount of protein in mg showing anticomplementary effect after heating	Percentage of aggregated material after heating	Gm-characters			Electrophoretic mobility	Contamination with normal γ
			Gm(a)	Gm(x)	Gm(b)		
Normal γ	0 09	15§	/?	—/?	+/+	normal γ	
M 196	0 016	10	+/+	+/+	—/?	γ 2 3	0
M 494	>0 50	0	+/+	—/—	—/—	γ 2 3	trace
M 559	0 016	0†	/?	—/?	—/?	γ 3	0
M 567	>0 50	0	/—	—/—	—/—	γ 1	0
M 610	>0 50	0	+/+	—/—	—/—	γ 3	0
M 611	0 008	45†	+/+	—/+	—/?	γ 2-3	0
M 612	0 016	0	+/+	—/—	—/?	γ 2 3	trace
M 619	>0 50	0	—/	—/—	+/+	γ 3	trace
M 627	0 25	0	?/?	—/—	—/—	γ 2	0

\+ indicates positive reaction — negative and ? intermediate reaction
Oblique separates reactions before and after heating
§ Solution opalescent after heating
† Precipitates formed during heating

The titre of the first component of complement (C 1) was determined by adding R1 to different amounts of the complement M-component mixtures Compared to the control C 1 was decreased and then most *markedly* when the heated samples of M components or normal "γ globulin had been incubated with complement

No correlation was found between the electrophoretic mobility of the M components and the anticomplementary effect appearing after heating Neither could this anticomplementary effect be ascribed to contamination of the M component preparations with normal γ globulin (Table 1)

No correlation could be observed between the Gm type of the native M components and *their anticomplementary* power (Table 1) The Gm characters of M components will be the subject of a future paper (*Nilsson*) Besides the increased anticomplementary power appearing after heating changes were observed in the Gm-characters of the normal γ globulin and of M components 196 559 611 and 612 Agglutination inhibiting activities not present in the solutions before heating appeared The changes of components 196 and 612 was at most slight while that of M-components 559 and 611 was substantial M component 627 previously grouped as Gm (a x b) showed an intermediate agglutination inhibiting activity in the Gm(a) test system also before

than 15 per cent of the protein Table 1 gives the electrophoretic mobility of the M components and their Gm types

Preparation of γ globulin from normal sera Pure normal 'γ globulin was isolated by precipitation with Rivanol and ammonium sulphate (*Horejsi & Smetana* 1956) and purified further by chromatography on DEAF cellulose The normal serum used was of Gm type (a x-b+)

The M components and the normal γ globulin were studied in native state and after heating at 63° C for 15 minutes

Barbital buffer containing calcium and magnesium ions in accordance with *Pillemer et al* (1956)

Sensitized sheep cells according to *Pillemer et al* (1956)

As *complement* was used a pool of human sera separated within 5 6 hours after sampling and stored at —60° C

METHODS

All the M components and the normal γ globulin preparation were dialyzed against the barbital buffer before tested for anticomplementary activity To tubes containing complement (fresh human serum, undiluted) were added aliquots of different dilutions of the M components and of the normal γ globulin respectively, and to a control tube aliquots of human complement and barbital buffer After incubation of the tubes at 37° C for 30 minutes the complement activity of the samples was determined by adding 1 ml of the sensitized sheep cells to varying amounts of the complement M component mixtures The haemolytic activity was estimated after incubation of the tubes at 37° C for 30 minutes and was expressed as 50 per cent haemolytic units (CH_{50}) The lowest amount of protein capable to reduce 12 CH_{50} to less than 1 CH_{50} was regarded as anticomplementary

Titration of C 1 was performed on the different mixtures by the technique given by *Pillemer et al* (1956) using an R1 prepared from pooled human sera

Gm determination was performed according to *Harboe & Lundevall* (1959) Aliquots of M components adjusted to equal protein concentration were added to

of the rheumatoid test serum

ed in the various Gm systems

were made at the same time

the agglutination score The samples were diluted from a protein content of 1 per cent irrespective of heat precipitated protein

Aggregation of gammaglobulin γ globulin (1 per cent) dissolved in a solution containing 3 parts physiological saline and 1 part M/15 phosphate buffer (pH 7.2) was heated at 63° C for 15 minutes The solution was then rapidly cooled to room temperature

The degree of aggregation could be roughly estimated a) from the appearance of opalescence or cloudiness and precipitation of protein and b) more quantitatively by preparative zone ultra centrifugation (*Kunkel* 1960)

RESULTS

Assessment of Anticomplementary Effect

Amounts of 0 25 mg and less of the unheated M-components and normal γ-globulin did not reduce 12 CH_{50} to less than 1 CH_{50} With the largest amount tested, 0 5 mg, anticomplementary effect was shown for M 611 and 612, but not for the other preparations

Heating markedly increased the anticomplementary effect of normal "γ"-globulin so that effect was obtained with an amount of 0 09 mg The anticomplementary activity of the different M-components varied considerably after heating In four of them this activity exceeded that of normal "γ' globulin, the other 5 were clearly less anticomplementary

Heating of the normal γ globulin and of the M components induced a varying degree of aggregation (Table 1) M components 559 and 611 formed complexes which precipitated during the heating procedure The normal γ globulin preparation became opalescent Ultracentrifugation of the solutions after they had been heated revealed considerable amounts of aggregated material in the normal γ globulin preparation and in the solutions of M components 196 and 611 That the solutions of M components 559 612 and 627 also contained aggregated γ globulin not demonstrable by the method of centrifugation used is possibly suggested by their changed reaction after heating in the Gm tests—soluble aggregates have a marked affinity for the rheumatoid factor (*Franklin et al* 1957) Nothing suggested aggregation in any of the other M components after exposure to heat

The 3 aggregated M components had a high isoelectric point but they did not differ in this respect from several of the other non aggregating M components

Olhagen (1945) noted that anticomplementary power was related to the appearence of opalescence on heating of strongly anticomplementary sera and that the anticomplementary effect was reduced or disappeared on filtering of the sera through Chamberland filter L 3 or Jena Glass Filter 11G5 He also found that the effect of weakly anticomplementary sera in which no opalescence was observed on heating vanished after such filtration *Norgaard* (1955) described an experiment where the anticomplementary titre of a heated strongly anticomplementary serum after centrifugation at about 40000 G for 1 hour fell considerably in the upper layer of the tube *Norgaard* also described that he has found strongly anticomplementary sera not showing opalescence on heating Normal sera which had been heated until they have become strongly opalescent exhibited only a very slight anticomplementary effect

Recently *Frommhagen & Fudenberg* (1962) found that normal 7S γ globulin on heating at 63° C for 20 minutes showed a *sharp increase* in opalescence and in anticomplementary activity Aggregates of a size of 100S 1000S prepared from such heated 7S γ globulin was found to be highly anticomplementary

Of the 4 M components exerting a strong anticomplementary activity after heating 2 contained considerable amounts of aggregated material (196 and 611) *whereas this* was only suggested in the other two (559 and 612) by their changed reaction patterns in the Gm test It cannot be excluded that aggregates capable of fixing complement may also have been present in the heated solutions of M components 559 and 612 Compared to the other two components the concentration of aggregated material must however have been lower

On the other hand M components showing no increase in anticomplementary activity on heating showed no aggregation or change of Gm characters

It appears reasonable to assume that the anticomplementary power

heating, which indicated a change in the preparation, possibly induced by repeated freezings and thawings

Heating had no demonstrable effect on the Gm-reaction pattern of any of the M-components lacking anticomplementary power (M 424, M 567, M 610, M 619)

Heating at 63° C for 15 minutes induced a varying degree of aggregation of the M-components (Table 1) Before the solutions were heated they were clear and ultracentrifugation revealed no soluble aggregates After being heated the solution of normal "γ"-globulin became slightly opalescent and in solutions of M-components 559 and 611 protein was precipitated Ultracentrifugation of the solutions after they had been heated showed that about 15 per cent of the normal "γ"-globulin, about 10 per cent of M-component 196 and about 45 per cent of M-component 611 consisted of aggregated material No rapidly sedimenting material could be demonstrated in the solutions of the other components after heating This also holds for the clear supernatant of M-component 559

No aggregates were formed in any of the solutions of the M-components in which heating produced no increase of the anticomplementary effect Of the M-components which increased in anticomplementary effect on exposure to heat two (M 196 and M 611) formed soluble aggregates, as the normal "γ"-globulin preparation did The M-component 559 precipitated on heating, but ultracentrifugation of the strongly anticomplementary material remaining in solution did not reveal any aggregates Neither did M-components 612 and 627 show aggregates after heating

Thus no absolute correlation was found between the anticomplementary capacity of the M-components and the content of soluble aggregates demonstrated in the preparations

DISCUSSION

The anticomplementary effect of normal γ-globulin which increases markedly after heating or on adsorption to bentonite could be ascribed to an inactivation of C'1, C'4 and C'2 by the aggregated γ-globulin analogous with the complement fixation by antigen-antibody aggregates (*Ishizaka & Ishizaka* 1959, *Christian* 1961, *Marcus* 1961)

In the present work it was found that, after heating 4 out of 9 highly purified M-components had a stronger, and the remaining 5 a weaker anticomplementary effect than heated purified normal "γ"-globulin

Only one "γ"-globulin preparation obtained from a single normal serum was studied It is therefore not possible to say whether individual "γ"-globulin preparations from healthy subjects generally have an anticomplementary activity of the same strength as that found here

The inactivation of C'1 on incubation of complement with M-components indicated that the anticomplementary effect of the M-components is of the same nature as that of normal "γ"-globulin

FROM THE INSTITUTE OF VIROLOGY, UNIVERSITY OF UPPSALA, UPPSALA, SWEDEN

INHIBITORY EFFECT OF AMINOPTERIN AND 5 BROMODEOXYURIDINE ON THE MULTIPLICATION OF SIMIAN VIRUS 40

By

HANS DIDERHOLM

Received 22.1.63

It is known that aminopterin and 5 bromodeoxyuridine (5 BDU) interfere with the synthesis of normal DNA in animal cells (*Ait et al* 1958, *Smith et al* 1960 and *Simon* 1961). Viral DNA synthesis is most probably also affected by these substances, as they inhibit the synthesis of DNA viruses but not RNA viruses. Thus *Smith et al* (1960) found that the multiplication of polyoma virus, a DNA virus, was inhibited by aminopterin and 5 BDU in mouse embryo cell cultures whereas the RNA containing encephalomyocarditis virus was not affected. *Herrmann* (1961) showed that 5 BDU had an inhibitory effect on the DNA containing vaccinia and herpes simplex viruses in chick embryo cell cultures while the RNA containing West Nile and Newcastle disease viruses were not inhibited. In the study of *Simon* (1961) it was found that in HeLa cell cultures aminopterin as well as 5 BDU inhibited the vaccinia virus but not the RNA containing Newcastle disease and poliomyelitis viruses.

The present report deals with the effect of aminopterin and 5 BDU on the multiplication of simian virus 40 (SV_{40}) in monkey kidney cell cultures. This virus has recently been isolated from kidney cell cultures of rhesus and cynomolgus monkeys and it has been isolated from certain lots of virus vaccines prepared from such cultures (*Sweet* & *Hilleman* 1960). The virus has thus been given to many thousands of persons without adverse effects being reported, but SV_{40} given to newborn hamsters is able to induce sarcomas (*Girardi et al* 1962 and *Eddy et al* 1962).

MATERIAL AND METHODS

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This investigation was supported by grants from the Swedish Cancer Society.

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FROM THE INSTITUTE OF VIROLOGY, UNIVERSITY OF UPPSALA, UPPSALA, SWEDEN

INHIBITORY EFFECT OF AMINOPTERIN AND 5-BROMODEOXYURIDINE ON THE MULTIPLICATION OF SIMIAN VIRUS 40

By

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It is known that aminopterin and 5-bromodeoxyuridine (5-BDU) interfere with the synthesis of normal DNA in animal cells (*Kit et al* 1958, *Smith et al* 1960 and *Simon* 1961). Viral DNA synthesis is most probably also affected by these substances, as they inhibit the synthesis of DNA viruses, but not RNA viruses. Thus *Smith et al* (1960) found that the multiplication of polyoma virus, a DNA virus, was inhibited by aminopterin and 5-BDU in mouse embryo cell cultures whereas the RNA containing encephalomyocarditis virus was not affected. *Herrmann* (1961) showed that 5-BDU had an inhibitory effect on the DNA-containing vaccinia and herpes simplex viruses in chick embryo cell cultures while the RNA containing West Nile and Newcastle disease viruses were not inhibited. In the study of *Simon* (1961) it was found that in HeLa cell cultures aminopterin as well as 5-BDU inhibited the vaccinia virus but not the RNA containing Newcastle disease and poliomyelitis viruses.

The present report deals with the effect of aminopterin and 5-BDU on the multiplication of simian virus 40 (SV_{40}) in monkey kidney cell cultures. This virus has recently been isolated from kidney cell cultures of rhesus and cynomolgus monkeys and it has been isolated from certain lots of virus vaccines prepared from such cultures (*Sweet & Hilleman* 1960). The virus has thus been given to many thousands of persons without adverse effects being reported, but SV_{40} given to newborn hamsters is able to induce sarcomas (*Girardi et al* 1962 and *Eddy et al* 1962).

MATERIAL AND METHODS

Subcultures were made from primary kidney cell cultures of the African green monkey *Cercopithecus aethiops*. About 4×10^{6} cells in 10 ml Hanks solution with 0.5 per cent lactalbumin hydrolysate and 10 per cent heat inactivated calf serum were seeded into 4-ounce prescription bottles.

When monolayers had been formed the cultures were infected with 10^{4} $TCID_{50}$ of

This investigation was supported by grants from the *Swedish Cancer Society*.

SV_{40} A strain that could be neutralized by antiserum to strain 776 was used (The virus was kindly supplied by Dr *D J Magrath*, Research Council Laboratories London) It had had a number of passages in our laboratory in kidney cell cultures of the African green monkey

After adsorption for 1 hour at 37° C the cultures were washed with phosphate buffered saline (PBS) to remove unadsorbed virus, and Parker's solution with 2 per cent heat-inactivated calf serum and inhibitor was added Aminopterin (Mann Research Laboratories, New York) as well as 5-BDU (Schwartz Bioresearch New York) were used in the concentration of 20 µg per ml All media contained 100 units of penicillin and 100 µg of streptomycin per ml

Virus was harvested at 4, 8 12 and 16 days after infection The supernatant was removed and fresh medium containing inhibitor was added To some cultures 1 ml PBS was added instead of medium, after which they were frozen and thawed three times to liberate the intracellular virus

The titrations were performed in kidney cell cultures of the African green monkey The medium consisted of Parker's solution and 2 per cent heat-inactivated calf serum Serial tenfold dilutions of the virus were prepared and 0 1 ml of each dilution was inoculated into each of 3 tubes which were incubated in the rolling position Readings were made after 14 days and the ID_{50} was determined according to *Kärber* (1931)

The effect of aminopterin and 5 BDU on the multiplication of poliomyelitis virus in kidney cell cultures of the African green monkey was also studied Virus of the LSc 2ab strain was used and each culture received 10^2 $TCID_{50}$ The culture material and methods were otherwise identical to those described above The concentration of inhibitor was also the same as in the SV_{40} study To get a more adequate comparison with the results obtained with SV_{40} cultures of different ages and exposed to the inhibitor for different lengths of time were used The virus was harvested at 2 days after infection The amounts of virus in the supernatant and in the cells were assayed in the same manner as described above with the exception of the composition of the medium and the time for readings Parker's solution without serum was used and readings were made after 7 days

The number of cells in untreated cultures and in cultures treated with inhibitor was also estimated The material methods and the concentration of inhibitor were the same as in the experiments with infected cultures After trypsinization the cells were washed by centrifugation diluted and counted in a Burker chamber

RESULTS

The results obtained with SV_{40} are given in Figs 1 and 2 In Fig 1 the titres in the supernatant are given while Fig 2 shows the titres in the cells It is seen that the multiplication of SV_{40} is suppressed by the addition of aminopterin and 5-BDU This effect is most pronounced with 5 BDU, no virus was detected in the supernatant at any time and only low titres were obtained in the cells

There was also an inhibitory effect on the cytopathic changes caused by SV_{40} Cultures without inhibitor showed typical cytoplasmic vacuolation in a few cells at 4 days after infection, and, after 16 days, most of the cells showed vacuolation In cultures with aminopterin, cells with vacuolation were first seen 7 days after infection and their number thereafter increased only slightly Vacuolated cells were rarely seen in cultures with 5 BDU

No inhibitory effect was obtained in parallel experiments with poliomyelitis virus All cultures had at the time of harvesting about the same degree of cytopathic changes

The cell counting showed no differences between the number of cells in untreated cultures and in cultures treated with inhibitor

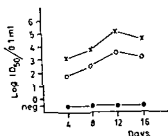


Fig 1

SV_{40} titres in the supernatant after the addition of aminopterin and 5 BDU

O———O with aminopterin
●———● with 5-BDU
×———× without inhibitor

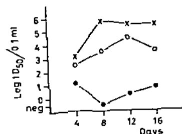


Fig 2

SV_{40} titres in the cells after the addition of aminopterin and 5 BDU

O———O with aminopterin
●———● with 5-BDU
×———× without inhibitor

DISCUSSION

As mentioned in the Introduction there is evidence that aminopterin and 5 BDU not only interfere with the normal DNA synthesis of animal cells but also with the viral DNA synthesis It has been shown that a number of DNA containing viruses are inhibited by these substances while they have no effect on RNA containing viruses (*Smith et al* 1960, *Herrmann* 1961 and *Simon* 1961)

The results of this study show inhibitory effect on the multiplication of SV_{40} but no effect on the multiplication of the RNA containing polio myelitis virus or on the cell count This indicates that the type of nucleic acid present in SV_{40} in DNA

The interpretation that the nucleic acid of SV_{40} is DNA agrees with *Gerber*s (1962) findings suggesting that phenol extracts from SV_{40} contain infectious DNA and also with the results obtained by the aid of acridine orange staining (*Mayor et al* cited by *Melnick* 1962)

SUMMARY

The effect of aminopterin and 5 bromodeoxyuridine (5 BDU) on the multiplication of simian virus 40 (SV_{40}) in monkey kidney cell cultures was studied It was found that these substances were effective inhibitors Since they are known to affect the synthesis of viruses containing DNA but not RNA the results indicate that the nucleic acid of SV_{40} is DNA

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FROM THE DEPARTMENT OF CLINICAL CHEMISTRY UNIVERSITY HOSPITAL
UPPSALA SWEDEN

HUMAN COMPLEMENT COMPONENTS IN STARCH GEL ELECTROPHORESIS

1 *Lability of Some Starch Gel Electrophoretic Serum Protein Fractions to Heat, Storage and Decomplementation*

By

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Received 23 11 63

Several attempts have been made to isolate the different complement factors from serum If, however, the criterion of high protein purity is demanded, none has as yet been isolated with the exception of one of the C 3 components (6, 7, 8)

In 1941 *Pillemer et al* (1) prepared two fractions with complement activity from human serum by means of ammonium sulphate precipitation One of the fractions, the "mid piece" with C'1 activity, had a carbohydrate content of 2 7 per cent and a mobility of 2 9 $\times$ 10 ^{5} in free electrophoresis with phosphate buffer at pH 7 7, ionic strenght 0 2 The other fraction, the 'end piece', with an activity of both C'2 and C'4 had an electrophoretic mobility of 4 2 $\times$ 10 ^{5} and contained 10 3 per cent carbohydrate *Lepow et al* (2), by a precipitation and extraction procedure, isolated a C 1 active fraction from serum *euglobulins* The preparation involved a 30–50 fold purification in relation to the serum, but it was heterogeneous on ultra centrifugation *Borsos* (3) isolated a "functionally pure" *C'2 fraction* from guinea-pig serum by means of ion exchange chromatography The preparation, however, still contained large quantities of other serum *proteins* *Arday & Pillemer* (4) isolated C'3 by further fractionation of a Cohn-fraction (III–1 Method 9) The isolated material was, however, electrophoretically heterogeneous In 1959 *Rapp et al* (5) separated two C'3 components, which they called C'a and C'b, from guinea pig serum by ion exchange chromatography, these components were together able to convert the complex EAC'$_{142}$ to E* In 1961 *Taylor & Leon* (6) demonstrated three C'3 components called C'3a, C 3b and C'3c, also by ion exchange chromatography, in both human and guinea-pig serum Up to the present time there are no physico chemical data regarding these components

In 1960 *Müller Eberhard et al* (7, 8) isolated a glycoprotein from human serum by means of anion exchange chromatography, this glyco

protein was named β_{1C} after its immuno-electrophoretic behaviour This was the first time that a complement factor was produced in a completely pure form both from a serological and a protein-chemical aspect Later studies have shown it to be probable that β_{1C} is identical with the C'3a of *Leon*'s nomenclature (9, 6). β_{1C} had the ability to substitute guinea-pig but not human R3 on immune haemolysis, and was able, further, to impede the decay of the EAC'$_{1,4,2}$-complex to EAC'$_{1,4}$ at 37° C The protein, which had a sedimentation coefficient (S_{20W}^0) *of 9 5, became changed on storage to an immune-haemolytic* inactive form with a sedimentation coefficient of 6 5 This inactive form could also be isolated in a completely pure state from old plasma, and was called β_{1A} after its immuno-electrophoretic behaviour. Both the active and the inactive forms contained about 2 7 per cent carbohydrate Thus of the known C'factors only (β_{1C}) can at present be visualized as a single electrophoretic fraction

Smithie's introduction of electrophoresis into starch gel (10) has increased our knowledge of serum proteins, and especially of those with genetic variants (10 19) By means of two-dimensional electrophoresis, 22 distinct fractions can be distinguished in human serum (20), some of which are still not identified as regards their function (21) A number of so-called alpha-beta-globulins, which migrate after transferrin, belong to this category *Poulik*'s modification of *Smithie*'s method with a discontinuous buffer system (22) gives a very distinct separation and also has the advantage of a shorter electrophoretic period of only 3–4 hours

On electrophoresis of human serum with the latter method two zones stainable with amido black 10 B are regularly observed in the region behind transferrin One or more subfractions may be concealed in these stainable zones, but reference will only be made in the following to what is immediately evident for the eye, namely two bands or fractions Preliminary experiments showed that these fractions were heat-labile, especially the slower moving one Since complement is typically heat labile, a detailed study has been made of the possible relationship between these electrophoretic components and complement Changes *in the two fractions, occurring both after storage or treatment of* serum with immune aggregates, have also been studied The possibility of such a relationship to complement is supported by the fact that *Fischel & Pearlman* (23) established C'1, C'2 and C'3 activity in alpha-beta-globulin fractions obtained by continuous flow paper electrophoresis of both guinea-pig and human adult serum They also found that the alpha-beta-globulins occur in low concentration in umbilical cord serum from the newborn compared with the content of these proteins in the maternal blood The reduced alpha-beta-globulin content was correlated at the same *time with a complement titre in the umbilical cord serum* 50 per cent lower than in the mother

Electrophoretic investigations of heat-treated serum have been car-

ried out previously by *van der Scheer et al* (24) They showed with free electrophoresis that horse serum which had been heated to approximately 65° C was denatured with the formation of a "colloidal component C" which moved approximately in the beta globulin region This arose chiefly at the expense of the globulins, but was also able to remove considerable quantities of albumin The "C" substance was not produced by the heating of albumin which had been isolated by 50 per cent saturation with ammonium sulphate It was, however, produced when isolated globulin was heated In the same year *Kleczkowski* (25) showed that the heating of an antiserum euglobulin fraction in the presence of other serum fractions led to a change of the antibodies, in such a way that they could be coupled to, but not flocculate their antigens When the euglobulin fraction was heated in the absence of other serum fractions, such changes in the properties of the antibodies did not occur He considered the product that was formed in the latter case to be a complex of two different proteins, which became bound to each other during denaturation The problem regarding protein protein interaction has been re attacked by *Schultz* (26), among others, with paper electrophoretic studies of heat treated human serum He established the occurrence af a single composite band ("C" component) which appeared to be due to the fusing of the alpha₂- and beta-peaks He left the question open as to whether this component comprises a protein protein complex or is the result of a partial denaturation of one or more proteins with a resulting changed electrophoretic mobility

MATERIAL AND METHODS

Sera and Plasma Blood obtained from healthy donors and from umbilical cord was allowed to coagulate at rom temperature for 30 minutes and then stand at +4° C for 2 4 hours before separation of the ser [illegible]

[illegible] diphtheria antiserum (horse) and tetanus toxin antitetanus serum (horse)[1] When the equivalence relationship between antigen and antibody had been determined the antigen [illegible] tes [illegible] was [illegible] at [illegible] at [illegible] (8) In the preliminary experiments the quantities of immune aggregates required to obtain a sufficient [illegible] titre of the ser [illegible] veronal

[1] Kindly placed at my disposal by *Dr Carola Neumüller* State Bacteriological Laboratory Stockholm

[2] Prepared immune aggregates were kindly made available by Dr B Hederstedt State Bacteriological Laboratory Stockholm

[3] Sheep's blood was obtained from the Bacteriological Institute Uppsala University

buffer containing Ca and Mg ions (28), and sensitized for 10 minutes at 37° C with rabbit amboceptor against Forsman antigen. The preparation of antigens and the immunization of the rabbits were carried out according to *Rapp's* method (29).

Complement reagents were prepared from fresh pooled serum. R1 and R2 were prepared by means of gel filtration[4] (30) and R3 and R4 in the conventional way by treatment with zymosan and hydrazine respectively (31, 32). RP serum (serum devoid of properdin) was prepared in accordance with *Pillemer et al.* (33). The reagent criteria, the mode of procedure in immune haemolysis and the calculations of the complement titres have been described previously (30).

Starch. Potato starch was in part hydrolyzed in this laboratory (Aroostokrat starch from A/B Rudolph Grave, Stockholm) and in part obtained ready hydrolyzed from the Connaught Medical Research Laboratories, Toronto, Canada (Lot Nos. 158 and 160). Different types of starch vary considerably in their separation ability. Several factors are of importance for any one type of starch, such as the length of the hydrolysis period, the relation between quantity of starch and the buffer volume and the degree of gelification (10, 34). The ionic strength of the buffer used in the preparation of the starch gel is also important (20). Great diffi

ducible

e starch

in this

respect. Firstly the ionic strength in the gel buffer. Lots 158 and 160 gave better separations when the ionic strength of the gel buffer was increased by 10–15 per cent compared with that originally used by *Poulik* (22). The second and even more important condition concerned the procedure in boiling the gel. In the preparation of gel with lots 158 and 160 it was necessary to continue boiling with evacuation of air by suction until the gel became sufficiently liquefied. If this latter condition was fulfilled satisfactory separation was always obtained.

Electrophoresis in starch gel was carried out by *Poulik's* method based on the principles laid down by *Smithies* (10), *i.e.* with a discontinuous buffer system (22). The gel was prepared with tris-citrate buffer with a pH of 8.65. A suitable ionic strength was ascertained for each type of starch. The electrode vessel contained 0.3 M boric acid and 0.05 M sodium hydroxide. The gel was cast in a plastic vessel measuring 0.6 × 12 × 16 cm. Whatman 1 filter papers of approximately 5 × 8 mm were saturated with serum and inserted into a slit a third of the distance measured from the cathode side. The site of application was covered with petroleum jelly and the entire gel with plastic foil. Communication with the electrolyte vessel was established by means of three Whatman 1 filter papers. The electrophoresis was performed at +4° C with a voltage of 6V/cm. 6–8 sera were separated simultaneously in each experiment and a serum with an excess of haemoglobin was also included in each run. When the free haemoglobin of this haemoglobin saturated serum had moved 37 mm from the starting point the electrophoresis was terminated. In this way evaluation of the results was facilitated. Immediately after the end of electrophoresis the gel was cut horizontally with a thin piano string and one half was stained for 30–45 seconds in amido black 10 B dissolved in alcohol, acetic acid and water in the proportions of 50/10/50 (V/V) (10). The gel was then destained with the above solvent solution. The other half of the gel was used when required for haptoglobin group determinations.

Since the complement factors occur in very low concentrations in the serum tests were first made to find out if the method selected was sufficiently sensitive to demonstrate the presence of such quantities. The quantity of C' nitrogen was estimated at 0.03–0.05 mg/ml serum (35) or recalculated in terms of protein 0.2–0.3 mg/ml serum, *i.e.* approximately 0.5 per cent of the total serum protein. Electrophoresis of highly purified albumin and transferrin[5] gave distinctly visible bands with concentrations as low as 0.125 mg protein/ml. Assuming that the stainability of the complement is just as great as that of albumin and transferrin it seems reasonable to suppose that the complement can be distinguished by the starch gel electrophoretic method.

Preparation of β_{1C}. This was prepared on DEAE Sephadex and DEAE cellulose anion exchange in accordance with *Müller-Eberhard et al.* (7). The β_{1A} protein was obtained by storing β_{1C} at + 4° C for 2–3 months.

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4 Sephadex was kindly given by *AB Pharmacia, Uppsala, Sweden.*
5 Made available by *AB Kabi, Stockholm.*

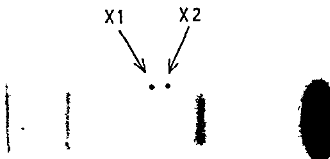


Fig 1
Starch Gel Electrophoretic picture of a normal human serum showing the two thermolabile fractions X1 and X2

RESULTS

A *Investigations on Fresh Serum and Plasma*

On electrophoresis of fresh normal human serum or plasma two bands are regularly observed in the region behind transferrin (Fig 1), and these bands were found in the subsequent investigations to be, inter alia, particularly labile to heat treatment and storage The foremost of these bands may be clearly observed in sera of Hp group 2 2, but is narrower than in sera of Hp groups 1-1 and 2-1 In sera belonging to these latter haptoglobin groups there is a haptoglobin band in exactly the same place in the electropherogram, which is lacking in sera of Hp group 2 2 It is probable that the component which was observed in the same place in Hp 2 2 sera is also concealed in this haptoglobin band Since, however, as far as we know the latter component in the Hp 2-2 sera is not connected with the haptoglobins, it has been considered justifiable to assume that it is present in all sera regardless of the haptoglobin group, but is concealed in the haptoglobin band in the sera of Hp groups 1-1 and 2-1 Regardless of these circumstances this band will be subsequently called X2

The other component in the electropherogram which moves more slowly is free from the haptoglobins, and this band will be subsequently called X1

The question of the relationship between X2 and X1, on the one hand and the haptoglobins and complement on the other, has been partially solved by comparative studies of sera from the foetus, newborn and adult X2 and X1 were found to be lacking in umbilical cord serum from 15 20 cm long foetuses, and the activity of the four complement factors was hardly measurable with immune haemolytical methods

TABLE 1

The Ontogenic Development of X1 and X2 Complement and Haptoglobin in Sera from the Foetus the Newborn and Adults

	Foetus	Newborn	Adult
Per cent Complement	≧ 0	50	100
X2	0	+	+
X1	0	+	+
Haptoglobin	0	≥ 0	+

+ = fraction X1 and X2 visible and Haptoglobin present 0 = no visible band

TABLE 2

Complement Components in Untreated Hp 2 2 Serum and in the Complement Reagents from the Same Serum The Electrophoretic Behaviour of the Fractions X1 and X2 in this Serum and in the Reagents after Different Kinds of Treatment

X Serum and complement reagents	Content of complement components of X	Content of X1 X2 in A		Content of X1 X2 in A after treatment as below					
				Storage		Heat		Immune precipitate	
		X1	X2	X1	X2	X1	X2	X1	X2
Hp 2 2 serum	1 2 3 4	+	+	0	+	0	0	0	+
R1	2 3 4	+	+			+	+		
R2	1 3 4	0	+			0	0	0	+
R3	1 2 4	+	+			+	+		
R4	1 2 3	+	+			+	+		
RP	1 2 3 4	+	+			+	+		

+ = fraction visible 0 = no band in electropherogram

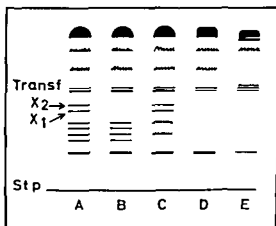


Fig 2

Schematic diagram of starch gel electrophoresis The main part of the gamma globulin region is excluded —Normal human serum type Hp group 2 2 (A) untreated (B) heated at 56° C for 10 minutes Cord serum from newborn infant (C) untreated (D) heated at 56° C for 10 minutes (E) untreated cord serum from 15 cm foetus

On the other hand both X1 and X2 were found in serum from the new born and at this period the haptoglobin has hardly yet developed (36), while the complement activity is approximately 50 per cent of that of the adult (23) This development of X1 and X2 from the foetal stage until birth is evident in comparisons of the electropherograms A C and F in Fig 2 It can also be seen that the haptoglobins are lacking at birth Since as far as is known there were no genetic variants in this region of the gel electropherogram this latter fact indicates that the component X2 is formed first and that in adults it is masked in the electropherogram by a haptoglobin band which is developed later during growth

B *The Influence on X1 and X2 of Different Treatments which Inactivate Complement*

1 *Storage*

If serum is stored at +2° C for 2 3 weeks and at 37° C for 3 10 days similar changes occur in serum from all three Hp groups X1 is eliminated from the electropherogram while X2 remains apparently quantitatively unchanged After these storage periods at both temperatures a titre reduction of 90 95 per cent was measured for all the classical complement factors C1 C2 C3 and C4 Thus serological inactivation occurs simultaneously with the disappearance of only one (X1) of the two bands

2 *Heating*

a) *Sera of Hp-group 2 1 and 1 1* X1 is eliminated from the electropherogram after only a relatively short heating period while X2 decreases only after a longer period of treatment or a higher temperature Fig 3 shows the amounts of complement remaining in sera heated for different lengths of time compared with the electrophoretic picture of these sera Fig 3 shows that after 20 minutes heating to 50° C the haemolytic titre of C2 and C3 decreased by 70 60 per cent At the same time X1 disappeared completely X2 still remained after 60 minutes heating but was somewhat more indistinctly separated than in untreated serum After this longer heating period all the C factors were almost completely inactivated It can also be seen from the figure that X1 gradually decreases with increasing heating periods while at the same time the stainability of the region of the β_{1C} (see Fig 7) increases At this treatment temperature no quantitative changes of protein fractions other than X1 and X2 are discernible with the eye but at higher heating temperatures (56° C and above) changes are less seen in other

b) *Sera of*

X2 disappe ... as X1 with treatment temperatures between 50° 56° C (see Fig 2 A and B)

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	Foetus	Newborn	Adult
Per cent Complement	≧ 0	50	100
X2	0	+	+
X1	0	+	+
Haptoglobin	0	≧ 0	+

+ = fraction X1 and X2 visible and Haptoglobin present 0 = no visible band

TABLE 2

Complement Components in Untreated Hp 2–2 Serum and in the Complement Reagents from the Same Serum The Electrophoretic Behaviour of the Fractions X1 and X2 in this Serum and in the Reagents after Different Kinds of Treatment

X Serum and complement reagents	Content of complement components of X	Content of X1 X2 in X		Content of X1 X2 in X after treatment as below: Storage		Heat		Immune precipitate	
		X1	X2	X1	X2	X1	X2	X1	X2
Hp 2 2 serum	1 2 3 4	+	+	0	+	0	0	0	+
R1	2 3 4	+	+			+	+		
R2	1 3 4	0	+			0	0	0	+
R3	1 2 4	+	+			+	+		
R4	1 2 3	+	+			+	+		
RP	1 2 3 4	+	+			+	+		

+ = fraction visible 0 = no band in electropherogram

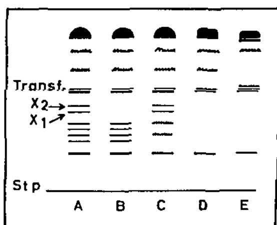


Fig 2

Schematic diagram of starch gel electrophoresis The main part of the gamma globulin region is excluded —Normal human serum type Hp group 2 2 (A) untreated (B) heated at 56° C for 10 minutes —Cord serum from newborn infant (C) untreated (D) heated at 56° C for 10 minutes (E) untreated cord serum from 15 cm foetus

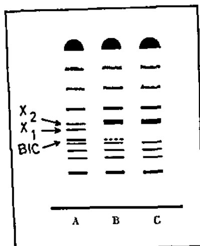


Fig 4

Starch gel electrophoresis of a Hp group 2 2 serum treated with ovalbumin anti ovalbumin precipitate
(A) untreated serum (B) a serum absorbed once with the immune precipitate and (C) a serum absorbed four times X1 has disappeared already after one absorption and X2 has increased The β_{1C} region is almost completely abolished after four absorptions

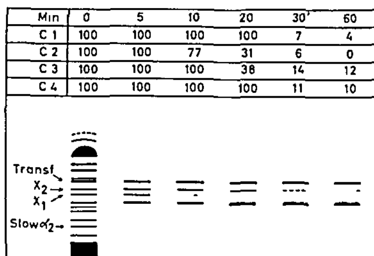
tions were performed with undiluted serum This is probably the reason that complete decomplementation was not attained

Fig 4 which illustrates decomplementation experiments with oval bumin antiovalbumin aggregate, shows that, visually, X1 was completely eliminated even at a 50 per cent complement decrease There was also a change in the X2 component which quite clearly increased in width, in these experiments Besides the changes in X1 and X2 a band at the site of β_{1C} also disappeared Incubation of a serum with immune aggregate in the presence of EDTA produced no reduction of X1 or change in X2 There was no decrease in either X1 or X2 with non specific adsorbents such as bentonite or kaolin

Decomplementation with immune aggregates is of great interest with regard to questions studied here The problem of complete decomplementation involved certain difficulties with reference to the conditions of the experiments It is clearly evident, however, that X1 is affected X2 is also altered but in a way which is difficult to interpret Non specific adsorption media do not appear to affect either X1 or X2

C *Studies of Complement Reagents*

The complement reagents R1, R2 and RP lack the complement factors C'1, C'2 and properdin respectively In the reagents R3 and R4 the factors C'3 and C'4 respectively are inactivated It might thus be expected that investigations of such reagents could provide information



Min	0	5	10	20	30'	60
C 1	100	100	100	100	7	4
C 2	100	100	77	31	6	0
C 3	100	100	100	38	14	12
C 4	100	100	100	100	11	10

Fig 3

Effect of heating on a Hp group 2-1 serum at 50° C

The tabulated figures refer to the titres of the complement components C 1 C 2 C 3 and C 4 in serum heated at 50° C for varying periods The values are given as per cent of those in untreated serum Below is given a schematic diagram of the corresponding electrophoretic pictures For the heat treated sera only the electrophoretic picture in the X region is given

c) *Umbilical cord serum* The heating of umbilical cord serum from the newborn also results in complete electrophoretic elimination of the fractions X1 and X2 (see Fig 2 C and D)

Heat treatment and storage thus give deviant results, the main difference lies in the lability of the X2 component with heating

3 *Decomplementation of Serum with Immune Aggregates*

Several decomplementation experiments were carried out with tetanus-antitetanus and diphtheria toxoid-diphtheria antiserum aggregates It was often difficult to attain obvious titre reductions of the complement factors with these immune aggregates, but in those cases where a titre reduction of more than 50 per cent was obtained for all C'-factors, subsequent electrophoresis of the treated sera showed that X1 had decreased or completely disappeared No changes were found in the X2 component—neither decreased nor increased stainability This was the case even with decomplemented Hp 2–2 serum, which lacks haptoglobin in the X2 region

Three decomplementation experiments of an Hp 2 2 serum were made using ovalbumin-antiovalbumin aggregates In the first experiment with only one absorption, a titre reduction for all complement factors of approximately 50 per cent was obtained, in the second experiment with three absorptions the titre reduction was somewhat larger, and in the third experiment with altogether four absorptions the reduction was about 75 per cent In order to avoid the effect of dilution on X1 and X2 in the electrophoretic experiments the absorp-

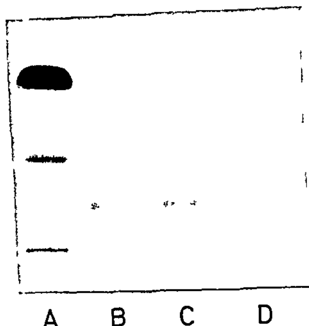
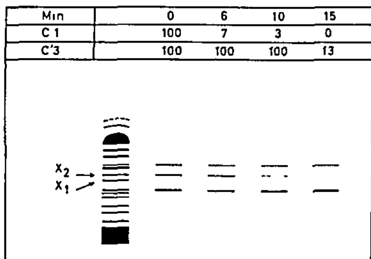


Fig 6
Treatment of R2 with ovalbumin antiovalbumin precipitate

(A) a normal serum of Hp group 2 2
(B) untreated R2 The titre of C'1 was 1200 H_{50}
(C) R2 absorbed once The C'1 titre was 400 H_{50}
(D) R2 absorbed twice The C'1 titre was 20 H_{50}

Electrophoretic investigations of R1 R3 R4 and RP showed that they all contained two fractions corresponding to X1 and X2 in whole serum On a few isolated occasions only one R1 fraction in the position of X1 was found in spite of the fact that the concentration of R1 was 1 5 times of whole serum This may probably be explained by the fact that the conditions for the electrophoretic separation of R1 were changed in relation to untreated serum Heat treatment of the complement reagents neither eliminated the electrophoretic X1 or X2 fractions nor was there any increased stainability of the β_{1C} region as was the case with heat treated whole sera Even when the reagents were heated for as long as 30 minutes at 56° C X1 and X2 still appeared unchanged After heating for 60 minutes the bands were clearly weaker, but there were also similar changes in other fractions of the electropherogram

Studies of complement reagents thus gave results which in certain respects deviated from those obtained with sera It was found in particular that in certain reagents X1 and X2 are more stable than in



Min	0	6	10	15
C 1	100	7	3	0
C'3	100	100	100	13

Fig 5

Effect of heating on a R2 reagent at 52° C.
The tabulated figures refer to the titres of the complement components C 1 and C'3 in untreated and treated R2 The values of the titres in the untreated R2 are set at 100 per cent Below is given a schematic diagram of the electrophoretic pictures

on which complement factors are localized to the X1 and X2 fractions

In this respect the investigations on the R2 reagent gave the most positive results R2, regardless of whether it is prepared by gel filtration or by dialysis against acetate buffer at pH 5 4 and ionic strength 0 02, and regardless of the Hp group serum from which it is prepared, has only one electrophoretic fraction in the region studied corresponding as to site with the X2 fraction in whole serum Even if the euglobulin precipitate is dissolved in a quantity of buffer giving a concentration 5 times as great as in the original whole serum, only this one fraction is seen in the region If such an R2 reagent is heated, this fraction, which corresponds electrophoretically to X2 of whole serum, always disappears Such an experiment is illustrated in Fig 5 After 10 minutes' heating to 52° C, electrophoretically only traces of the X2 fraction remain Immune-haemolytically, C'1 is almost completely inactivated after this heating period, while C'3 is not affected Decomplementation experiments with ovalbumin-antiovalbumin aggregate were also performed R2 was dissolved in veronal buffer containing Ca and Mg ions in optimal quantities In one R2 preparation the euglobulin concentration was 3 times higher than in the original serum One part was treated with immune aggregate for 45 minutes at room temperature and another part for two 45-minute-periods In Fig 6 it can be seen that in the last part C'1 had been almost completely eliminated (measured with the immune-haemolytical method), but in spite of this no definite decrease of either the X2 or any other fraction was observed in the electropherogram of this R2 preparation The same held for an immune aggregate-treated R2, where the euglobulin concentration was just as high as in the original serum

DISCUSSION

The main part of the results are arranged schematically in Tables 1 and 2, which may serve as a guide in the following discussion.

The investigations have provided a basis for the hypothesis that two heat-labile serum fractions X1 and X2, which in starch gel electrophoresis move in the alpha beta-region, are related to complement. Support for this hypothesis was obtained from

1) a correlation between X1, X2 and the complement activity in the ontogenetic development,
2) a correlation between X1 and complement with increased storage time of the serum,
3) a correlation between X1, X2 in an Hp 2-2 serum and complement on heat inactivation,
4) a correlation between X1 and complement on decomplementation of the serum,
5) a correlation between X2 and the complement activity on heat inactivation of R2.
6) To a certain extent the hypothesis is supported by the fact that the fractions X1 and X2 are found in R1, R3, R4, and RP.

Some of the results, however, are not unequivocally compatible with the assumption that both X1 and X2 contain complement, and these are

a) The storage of serum at +4° C and +37° C until all complement factors are practically entirely inactivated, eliminates X1 but not X2 from the electropherogram.
b) Heat treatment of R1, R3, R4 and RP does not eliminate either of the fractions X1 or X2.
c) Decomplementation of serum with immune aggregates reduces X1 but increases the X2 fraction.
d) Almost 100 per cent inactivation of C'1 by treating R2 with an immune aggregate did not produce any visible decrease in the X2 fraction of this R2.

Regarding point a) it may be stated that biological inactivation need not necessarily give rise to changed physico-chemical properties in proteins, *e.g.* altered electrophoretic mobility. The inactivation of complement to occur if serum is stored at +4° C and +37° C takes place under conditions which are *more* favourable than conditions of inactivation when serum is heated to 50°–56° C when X2 disappears, this is probably the reason why X2 is not eliminated from an Hp 2-2 serum stored at +4° or +37° C. A possible explanation may also be (not yet proved to the contrary) that X2 may be chemically inhomogeneous.

Point b). It is more difficult to *explain* why X1 and X2 in R1, R3, R4 and RP are not affected by heat treatment. It is possible that the preparation of these reagents involves treatment that produces suf-

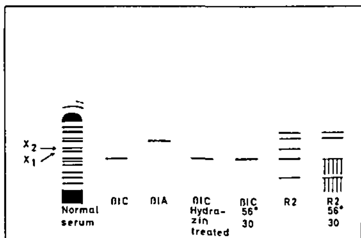


Fig 7

Electrophoretic studies of β_{1C} β_{1A} and R2 schemically reproduced
The electrophoretic mobility of isolated β_{1C} in starch gel is not changed after treatment with hydrazine or heat Contrary to this the β_{1C} band in a heated R2 is almost completely eliminated

sera One reagent, R2, contains only one of the X-components, namely X2 In this reagent X2 disappears from the electropherogram on heating but, as also in sera, it is not removed by decomplementation with immune aggregates

D *Studies of β_{1C} and β_{1A}*

It has been pointed out previously that β_{1C} is the only one of the complement factors that can as yet be isolated in, as far as we know, a completely pure form The same applies to inactivated β_{1C}, *i e* β_{1A} It is of interest whether these components are related to the fractions X1 and X2 It can be seen in Fig 7 that β_{1C} moves more slowly than X1 and β_{1A} more rapidly than X2

In order to elucidate the importance of the environment to proteins subjected to physico chemical treatment, β_{1C} was treated with heat and hydrazine *Muller-Eberhard* (37) showed that β_{1C} is extremely sensitive to hydrazine and quickly loses its complement activity with such treatment In Fig 7 it is shown that neither heat- nor hydrazine-treatment of β_{1C} affects its electrophoretic mobility If the whole euglobulin fraction (R2) whose principal fraction consists of β_{1C}, is heat treated, the β_{1C} band on the other hand is almost completely eliminated, and an increase of stainable material occurs in the region between the starting point and β_{1C}

Studies of β_{1C} and β_{1A} have thus shown that X1 and X2 do not contain either of these proteins Examples have also been shown of the fact that a protein in an isolated form does not change its electrophoretic mobility in starch gel after being subjected to certain physico-chemical treatment, but is sensitive to such treatment in the presence of other euglobulins

wholly dissociate from the aggregate, for example in analogy with the decay of EAC'1,4,2 to EAC 1,4 (28) This dissociated C'factor might then remain in the serum with either a changed or unchanged electrophoretic mobility As an example it may be mentioned that β_{1C}, which, as is known, becomes attached to immune aggregates (37), can nevertheless be demonstrated in the serum, however, now in the form of β_{1A} with a different electrophoretic mobility than that of β_{1C} (8)

It is thus evident that it is very risky to draw any definite conclusions regarding the correlation of complement and electrophoretic fractions from decomplementation experiments Neither the disappearance nor appearance of an electrophoretic fraction are therefore alone sufficient proof that the fraction in question does or does not contain complement It is thus still possible that X2 contains complement

It remains to comment on a few electrophoretic changes which were observed in connection with the investigations reported These include the increased stainability in the β_{1C} position (see Figs 3 and 7) This phenomenon is interpreted as due to denaturation products from the heat labile serum proteins On heating the complement reagents R1, R3, R4 and RP, when X1 and X2 were not eliminated, the phenomenon was not observed

The increased width of X2 in a serum that had been incubated with ovalbumin antiovalbumin precipitate may possibly be due to a change in physico chemical properties of some complement factor which had attached itself to the X2 fraction As a first guess, inactivated β_{1C}, i e β_{1A}, might be suggested To judge from the electrophoretic picture, β_{1C} has in the main disappeared from its place in the electropherogram It is true that it has been stated previously that β_{1A} moves considerably more rapidly than X2—with the fast alpha₂ (Fig 7)—but this applies to a β_{1A} which was the final product of about three months' storage of isolated β_{1C} at +4° C It cannot be precluded that β_{1A} may exist in different forms with varying electrophoretic mobility

Even if some of the individual results are thus difficult to interpret, the collected results constitute, however, fairly good support for the hypothesis that the electrophoretic fractions X1 and X2 contain C'protein Since both fractions in Hp 2-2 serum are rather heat labile, as is X2 in R2, and since both fractions are found in the reagents R3, R4 and RP which also contain the most heat labile complement factors, it may be provisionally postulated that X1 and X2 contain the factors C 1 and C 2 But it cannot be precluded, of course, that the other complement factors may also be included in X1 and X2 The only factor which with certainty is not included in X1 and X2 is β_{1C}, which in a purified form moves more slowly than X1

When, finally, it is attempted to determine which of X1 and X2 contains C'1 and C 2 respectively, the conclusions are based on the following reasoning Since C 2 is the heat labile component that is lacking in R2, and thus R2 only has one electrophoretic band in the

ficient physico-chemical changes to influence the electrophoretic separation in the starch gel It is obvious that we have here an example of the way in which the environment of the proteins determines the type of physico chemical changes to occur on their denaturation, which has been illustrated previously by *van der Scheer et al* and *Kleczkowski*, among others (24, 25) As a further example the inactivation of β_{1C}, both in a pure form and in the presence of other proteins, may be mentioned (Fig 7)

Protein denaturation is a problem of fundamental importance and the electrophoretic method is only one of many that can be used in its study The reason for the increased heat stability in the X1 and X2 fractions in the complement reagents R1, R3, R4 and RP can therefore hardly be discovered by means of the observations described above

It seems valid therefore to ascribe less importance to the results with complement reagents than to those with whole serum

Points c) and d) Decomplementation experiments of serum and R2 with immune aggregates showed that X2 was not eliminated electrophoretically in spite of the fact that the total C′ was reduced by 75 per cent in whole serum and C′1 almost completely in R2 as measured with the immune-haemolytical method In whole serum decomplemented in this way X2 had even increased in width and stainability, while X2 in the R2 reagent was unchanged One explanation could be that X2 does not contain complement at all In this connection it should be mentioned that the correlation between the immune-haemolytic activity of the complement factors and their protein substrate is not clear (38) Whether the C′1 and C′4 components, which immune-haemolytically have a much higher titre than C 2 and C′3, also represent a correspondingly higher amount of serum protein, is thus unknown Nor is it completely clear whether all the complement factors or only one or some of them interact to form an immune aggregate According to *Heidelberger et al* (35) the C′-nitrogen that is found in an immune aggregate consists mainly of C 1 This also was indicated by *Weigle & Maurer* (38) C′1 can also be eluted from $EAC'_{1,4}$ or $EAC'_{1,4,2}$ by the treatment of this complex with EDTA (39, 40) Since it has been shown that the rheumatoid factor combines with antigen antibody aggregates (41) and conglutinin with antigen-antibody complement complex (42) it is difficult to draw conclusions concerning the distribution of the different complement factors on this C′ nitrogen, from measurements of the C′-nitrogen attached to immune aggregates

In complete decomplementation of a serum, complement can no longer be demonstrated by the immune-haemolytical method after the immune aggregates have been centrifuged away This gives no information as to whether the complement factor or factors, which might attach themselves to the immune aggregate, do this completely or only partly Even if all the complement attaches itself there is a possibility that one or several of the complement factors, after fixation, partly or

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region, corresponding with X2 in whole serum, it can be concluded that X2 probably contains C′1 This is supported by the fact that after heat treatment R2 loses both its C′1 activity and also a fraction which as to position corresponds to X2 in whole serum In this case X1 should contain the other heat-labile component C′2 Final proof for or against the correctness of the conclusions drawn cannot be obtained, however, until the individual complement factors have been isolated It is also conceivable that the correctness of the hypothesis could be tested by investigating eluted protein fractions from starch gel-electrophoretically separated serum for the occurrence of complement activity This possibility is being tried out at present and preliminary results indicates that at least the assumption that C′2 is contained in X1 is correct

SUMMARY

With starch gel electrophoresis, two fractions belonging to the alpha beta-globulins and moving after the transferrin were observed in human sera In Hp group 1–1 and 2–1 sera the faster moving one contains a Haptoglobin band They were heat-labile, which motivated a study of whether they contained complement The fractions were lacking in umbilical cord sera from 15–20 cm long foetuses These sera also almost entirely lacked all complement factors Both fractions were observed in umbilical cord sera from the newborn Storage of the serum resulted in the disappearance of the more slowly moving fraction Heat treatment of the serum eliminated the slower fraction, and at higher temperatures and a longer heating period also partially the more rapid fraction from Hp-group 1 1 and 2–1 sera In Hp 2 2 sera both fractions disappeared after heating for a short period The complement reagents R1, R4 and RP contained both fractions, whose electrophoretic mobilities, however, were not influenced by heat treatment R2 contained only one fraction in this region corresponding as to position to the more rapidly moving fraction in whole serum, and this fraction disappeared after the heat treatment of R2 for a short period Decomplementation of serum with immune aggregates eliminated the more slowly moving of the two fractions

It seems probable from the observations described regarding (a) the lability of certain electrophoretic serum components on heating and storage and in connection with decomplementation and (b) the decrease in complementary activity in connection with such treatments, that certain complement factors can be localized to definite serum components Two fractions described here have exhibited particularly high degrees of lability and probable reasons have been given to support the hypothesis that factor C′2 is contained in the slower serum fraction and C′1, though more doubtful, in the rapid fraction

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THE EFFECT OF CROSS-LINKED DEXTRAN GEL (SEPHADEX®) ON HUMAN COMPLEMENT WITH SPECIAL REFERENCE TO THE THIRD COMPONENT (C'3)

By

KARL-ERIK FJELLSTRÖM

Received 4 iii 63

The cross linked large-molecular dextran gels (Sephadex®)[1] have found extensive application in the so called gel filtration procedure (see Ref 1 for review) In the gel filtration of serum for the separation of complement reagents (2) it was found that component C'3 was retarded in the column and at least partially separated from the other complement factors (see Fig 1) It was also observed that some inactivation of C'3 occurred either during the passage of serum through a column of dextran gel or when the gel was added directly to the serum

It is known that several large-molecular polysaccharides (3) including different dextran preparations (4, 5) can inactivate C'3 The first substance observed to have such an effect was fresh yeast (6), and *Pillemer et al* (7) found that this was due to an insoluble carbohydrate component in the yeast cell membrane, Zymosan These workers also studied the mechanism of this inactivation of C'3 It was dependent on the presence of Mg ions (8), on a hydrazine sensitive and heat labile serum factor (9) and on a serum protein, which they named properdin (10) The whole of this complex of factors they designated as the "properdin system" (11) They found that the reaction occurred *in* two stages First, at temperatures over 10° C (optimum 15–17° C) a complex between properdin and zymosan (PZ) was formed and this could inactivate C'3 at temperatures *exceeding* 17° C (optimum 32 37° C) The other complement factors were affected little or not at all by these reactions *Pillemer* concluded that *properdin* was a unique selective inactivator of C'3 and that it was not an antibody The correctness of this latter view, however, has been questioned (12, 13, 14, 15)

Among the polysaccharides *investigated* (including some lipopolysaccharides) the effects on complement differ very greatly In some cases C'3 is inactivated, in some only properdin is bound and C'3 is not inactivated, while others are wholly inactive (3) Up to now no

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for example, periodate Dextran gel thus seems to be a potentially suitable model system for studying the effect of high molecular weight polysaccharides on complement

The observation that C 3 is retarded during elution through a column of dextran gel motivated a study of the possibilities for utilizing this effect in the isolation of C'3 These experiments were, however, complicated by the simultaneously occurring inactivation The primary aim of the present work was therefore to study the factors operative in the inactivation The results obtained will be discussed in the light of earlier work with zymosan

MATERIAL AND METHODS

Sera Fresh serum either from individual donors or pooled was used in the preparation of complement reagents

Complement reagents R1 and R2 were prepared by gel filtration (2) R3 and R4 were prepared by treating serum with zymosan and hydrazine respectively at 37° C for 60 minutes (16) Compared with their original concentrations in serum the reagents as prepared were diluted as follows R1 1 3 R2 3 1 R3 1 2 and R4 1 1 5 The criterion for acceptance was that 0 20 ml of the reagents should not give haemolysis but that each when substituted in a haemolyzing system should give 100 per cent haemolysis

Immune haemolysis Sheep red cells were sensitized with amboceptor against the Forsman antigen prepared in this laboratory by the method described by *Rapp (16)* The total reaction vol me in the
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All comparisons between complement titres refer to experiments done on the same day and carried out with the same batch of sensitized sheep red cells the same complement reagents and by the sa
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study a reaction volume of only 1 25 ml
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the uncertainty in these quantitative studies several determinations of the value f each titre were made

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the next 4 5 hours under which conditions signs of spontaneous haemolysis were n t visible t the eye

Zymosan Zymosan (Fleischman Lot 7B 13)[1] was used in the preparation of R3 and in the comparative experiments with Sephadex

The dextran gels Sephadex[2] are large molecular polysaccharides containing 90 95 per cent alpha 1 6 glucosidic linkages. The remainder of the linkages are predomi

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[1] Zymosan (Lot No 7B 13) was kindly given by the Fleischmann Laboratories Standard Brands Incorporated New York

[2] Sephadex was kindly given by A B Pharmacia, Uppsala Sweden

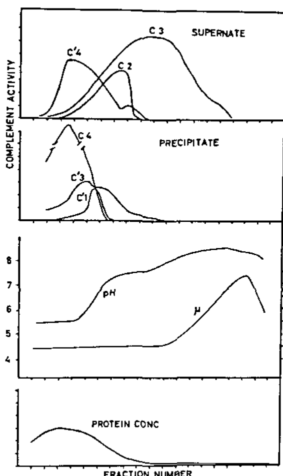


Fig 1

Preparation of R1 and R2 reagents by means of gel filtration
A column packed with Sephadex G 25 and equilibrated with acetate buffer at pH 5 3 and ionic strength 0 02 Fresh human serum was applied on to the column and eluted with the acetate buffer The euglobulin precipitate in every eluted fraction was washed twice with the buffer pH ionic strengtht and protein concentration were estimated in the supernatants The figure shows how the activity of the com ponents C 1 C 2 and C 4 coincide with the protein curve whereas the maximum of C 3 activity is retarded The complement activity is expressed as the ratio between optical density at 541 mμ of the haemolysate and optical density at 500 mμ of the protein (Folin method)

particular chemical structural property has been shown as the common factor among the active polysaccharides (3)

Zymosan consists mainly of carbohydrate in the form of polymerized glucose and also contains smaller amounts of nitrogen, phosphorus, magnesium and ribonucleic acid (16) It is a poorly defined substance as compared with dextran gel

The starting material for the preparation of a dextran gel is dextran which is synthesized by a bacterium (Leuconostoc mesenteroides) (1) The degree of cross-linking induced by epichlorhydrin can be controlled within a wide range (1), as can oxidation of the cross-linked gel with,

obtain the 1:16 dilution of serum usual in inactivation studies of C'3, in all three gel systems, 1.24 ml of buffer were added to G-25 since the V_0 for 0.1 gram G-25 was calculated as 0.26 ml. For 0.1 gram G-50, V_0 = 0.47 ml and only 1.03 ml of buffer was added to this tube. For 0.1 gram G-75, V_0 = 0.82 and the buffer volume added was 0.68 ml. In the table and accompanying text it is shown that these buffer additions give similar protein concentrations in all three tubes.

RESULTS

a) *Effect of the Time of Incubation on the Inactivation of C'3 with Sephadex*

Fig. 2 illustrates the effect on the C'3 titre in serum treated with G 50 at 37° C. The C'3 titre was measured with a zymosan preparation of R3 and with human $EAC_{1,4,2}$-*complex*. *The inactivation of C'3 by* Sephadex followed a similar time course to that with zymosan, and in this experiment was complete after about 60 minutes.

In a second series of experiments it was found that the inactivation time was to some extent related to the amount of gel, but also depended on the particular serum used. In some sera C'3 was thus completely inactivated after 35-40 minutes. For all sera so far studied it has been possible to inactivate C'3 completely with dextran gel.

TABLE 1

Correction of Serum Dilution with Regard to the Void Volume in the Suspensions of the Different Gel Types

Type of Dextran gel 0.1 gm of each	Serum ml	Void volume of 0.1 gm gel ml	Addition of buffer to achieve a dilution of 1:16	Total volume of diluent	Folin extinction (E_{750}) of diluted sample — Serum dilution 1:40	1:60	1:80	1:100
G 25	2.5	0.26	1.24	1.5	0.355	0.240	0.185	0.160
					0.345	0.240	0.180	0.150
G 50	2.5	0.47	1.03	1.5	0.325	0.240	0.185	0.160
					0.325	0.245	0.195	0.160
G 75	2.5	0.82	0.68	1.5	0.315	0.240	0.190	0.145
					0.340	0.240	0.175	0.150

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density wa ... spectrophotometer. The values show that similar protein concentrations were obtained for the three serum aliquots treated with the different dextran gel types and thus that the calculations of void volume and the buffer volume to be added, were correct.

nantly of the 1-3 glucosidic type (1) The water regain value (W_r), which is defined as the amount of water imbibed by 1 gram of any gel substance, is a rough measure of the degree of cross linking With increased density of cross linkages there is a reduced water regain Three commercially available types of sephadex have been used in these experiments, *i e* types G-25, G-50 and G-75 with respective W_r values of 2 5, 5 0 and 7.5 ml of water per gram. The gels used were prepared by pulverization of a block polymerisate and both dry and hydrated particles were thus highly irregular in form The dry particle size distributions used are described as fine (mainly between 37–75 μ), medium (mainly between 53–100 μ) and coarse (mainly between 100–300 μ).

Experimental study. 20 mg or more of dry dextran gel was as a rule used in the inactivation experiments, it was weighed on a balance with an accuracy of ± 1 mg The dextran gel grains were allowed to swell and were then washed twice in the same veronal buffer as was used in the haemolysis experiments After the second wash the grains were allowed to settle until a clear supernatant remained which was then sucked away. Because of the different elasticity of the differently cross linked gels centrifugation was not done but the grains were allowed to sediment spontaneously so as later to maintain the correct serum dilution (see below) The test serum and diluting buffer were then added The reaction between dextran gel and serum was allowed to take place in thin-walled round bottomed centrifuge tubes which were closed with corks covered with plastic film The tubes had previously been warmed in a water bath at 37° C for 4 minutes and then were rocked in a constant temperature room at 37° C Constant rocking is necessary otherwise the gel grains rapidly sediment to the bottom of the tube Other tubes containing zymosan instead of dextran gel, and also control tubes containing only serum, were rocked simultaneously In the treatment of serum at 16° C the centrifuge tubes were enclosed in a cylinder through which water at 16° C was circulated The whole cylinder with its contents was rocked continuously during the experiment

Calculation of Serum Dilution with Respect to the Void Volume of the Dextran Gel

According to *Gelotte* (20) the total volume of a gel system equilibrated with buffer or water can be described by the following equation

$$V_t = V_o + V_i + V_g \tag{1}$$

where V_t = total volume of gel bed (including interparticulate water)
V_o = outer or "void" volume (volume of fluid between the hydrated grains)
V_i = internal fluid volume (accessible to small molecular or ionic species)
V_g = volume of gel itself

V_i varies in relation to the total weight of gel and is related to the water regain (W_r(20) Dextran gel type G 25 which has the highest degree of cross linking at present available has a W_r value of about 2 5 ml of water per gram dry gel (W_r of G-50 = 5 0 ml and of G-75 = 7 5 ml) Since W_r and the weight of the substance (a) are known V_i can be calculated since

$$V_i = a\,W_r \tag{2}$$

V_t and V_g can be measured directly and thus V_o can be estimated V_o is important here because it is the volume into which the large molecular species of serum are diluted In the comparison between the effect of different amounts of gel on equal serum volumes this dilution factor must be taken into consideration The same consideration applies when gels of different degrees of cross linking are used The serum volumes must therefore be adjusted to a standard protein concentration by the addition of buffer

Table 1 summarizes an experiment in which 2 5 ml of serum were added to either gel types G–25, G–50 or G–75 which had previously been washed and equilibrated with buffer as described above In order to

obtain the 1:1 6 dilution of serum usual in inactivation studies of C'3, in all three gel systems, 1 24 ml of buffer were added to G-25 since the V_O for 0 1 gram G-25 was calculated as 0 26 ml For 0 1 gram G 50, V_O = 0 47 ml and only 1 03 ml of buffer was added to this tube For 0 1 gram G-75, V_O = 0 82 and the buffer volume added was 0 68 ml In the table and accompanying text it is shown that these buffer additions give similar protein concentrations in all three tubes

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G 25	2 5	0 26	1 24	1 5	0 355	0 240	0 185	0 160
					0 345	0 240	0 180	0 150
G 50	2 5	0 47	1 03	1 5	0 325	0 240	0 185	0 160
					0 325	0 245	0 195	0 160
G 75	2 5	0 82	0 68	1 5	0 315	0 240	0 190	0 145
					0 340	0 240	0 175	0 150

Three serum aliquots were mixed with equal quantities of dextran gels of three different degrees of crosslinking (G 25 G 50 and G-75) To obtain a constant serum dilution of 1 16 the total volume of buffer is 1 5 ml [illegible]

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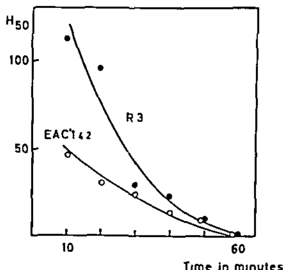


Fig 2

Effect of the time of incubation on the inactivation of C 3 with dextran gel Treatment of serum aliquots (2 5 ml) with 70 mg G 50 ml serum at 37° C. The incubation was stopped at 10 20 minutes etc and the serum titrated for residual C 3 both by zymosan prepared R3 and with human EAC 14 complex

Ordinate C3 activity in haemolysis units (H_{50})
Abscissa incubation time in minutes
Filled circles—titrated with R3
Open circles—titrated with EAC 142

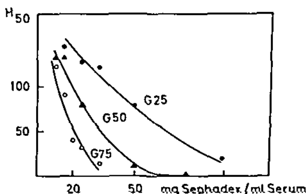


Fig 3

The effect of cross linking on inactivation of C 3 with dextran gel The titration of C 3 in serum treated with different amounts of dextran gels types G 25 G 50 and G 75 at 37° C for 60 minutes

Ordinate C 3 activity in haemolysis units
Abscissa mg dextran gel/ml serum
Filled circles serum treated with G 25
Triangels serum treated with G 50
Open circles serum treated with G 75

b) *Effect of the Amount of Dextran Gel and the Degree of Cross-Linking in the Inactivation of C 3*

Aliquots of serum were treated with increasing amounts of dextran gel types G 25, G 50 and G 75 The particle size of the three types were

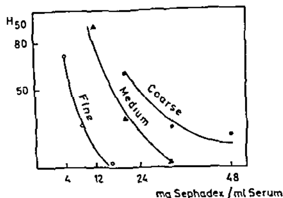


Fig 4

Inactivation of C'3 with dextran gel G 75 The effect of particle size
Serum aliquots were incubated at 37° C for 60 minutes with different amounts of dextran gel type G 75 of different particle sizes
Ordinate C'3 activity in H_{50} units
Abscissa mg G 75 ml serum
Closed circles G 75 coarse grade
Triangles G 75 medium grade
Open circles G 75 fine grade

essentially similar The results are shown in Fig 3, where it can be seen that complete inactivation of C'3 required at least twice the quantity of G 25 as G 50 and about twice as much G 50 as G 75 The results thus show that the ability of the gel to inactivate C'3 is apparently inversely proportional to the degree of cross linking, i e the smaller the number of cross-linking bridges the greater the C'3 inactivating effect

c) Effect of Particle Size of the Dextran Gel Grains on the Inactivation of C'3

Since the C'3 inactivating ability of dextran gels is proportional to the amount of substance used it might be expected that for any particular degree of cross linking a more finely particulate gel would have a greater effect than more coarsely divided material The effect of three different grain sizes of G 75 (lot To 7767) was compared From Fig 4 it can be seen that a given weight of "fine" grade gel is about twice as effective as the "medium" grade and about four times as effective as the "coarse" grade in the inactivation of C'3 There is probably thus a relationship between the effect of the gel and the total area of the gel particles

d) Effect of Temperature on the Inactivation of C'3 by Dextran Gel

In previous experiments serum was treated at 37° C Since zymosan, as previously mentioned, acts in a different way at temperatures below 10 C than at 15 17° C and 37° C, dextran gel was studied at the two

TABLE 2

Treatment of Serum with Different Amounts of Dextran Gel Types G 25 G 50 G 75 and Zymosan at +4° C for 17 Hours after which Time the Titres of the four Complement Components Were Measured and Compared with those in Serum Rocked without Dextran Gel (Control) Titres Are Given in H_{50} units

Mg ml serum	G 25		G-50		G 75		Zymosan		Control
	75	150	35	75	15	35	1 5	3 0	–
C 1	1280	1280	984	825	1280	691	894	940	1066
C 2	*200*	*220*	*200*	*200*	*213*	*173*	*206*	*206*	220
C 3	103	94	55	7	48	30	59	38	128
C 4	177	220	177	123	183	139	110	94	206

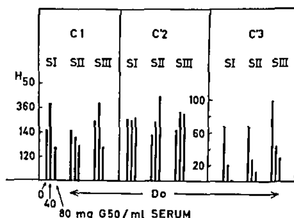


Fig 5

The treatment of three normal sera (S I II and III) with dextran gel type G 50 at +4° C for 38 hours The effects on the complement factors C'1 C'2 and C'3 are given

Ordinate Complement titres in H_{50} units
Abscissa mg G 50 ml serum 0 = no treatment

first-named temperature regions, i e at 4° C and 16° C Before each experiment tests were made to determine the smallest amounts of zymosan and dextran gel required to inactivate C'3 completely at 37° C in the same volume of serum as was used in experiments at +4° C and +16° C

1) *Treatment at +4° C* After treatment for 17 hours with increasing amounts of G 75 there was a clear reduction in C'3 but no definite lowering of C'4 The second component C'2 was not affected at all

In another experiment serum samples were treated for 17 hours with all three gel types G 25, G-50 and G-75 and also zymosan Table 2 shows that none of the titrated C'factors were affected by G 25 C'3 was significantly lower after treatment with G 50, G 75 and zymosan The exact values of the reductions in C'1 and C'4 are somewhat uncertain C'2 was not affected by any of the treatments

In a third experiment three different normal sera were treated with the gel type G-50 at 4 C for 38 hours In Fig 5 it can be seen that C'1

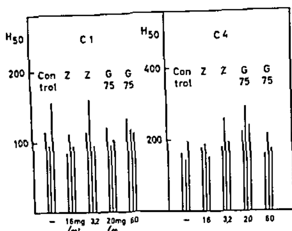


Fig 6

Treatment of serum with zymosan and dextran gel in the presence of Na3 EDTA
The effect on C'1 and C'4

The titration of C 1 and C4 in sera treated with zymosan (= Z in the fig) or dextran gel for 60 minutes at 37° C The minimal amount of zymosan and G 75 necessary for complete inactivation of C 3 in 1 ml serum was 1 6 mg and 20 mg respectively Serum samples were also treated with twice the minimal amount of zymosan and three times the minimal amount of G 75 The titres are given in H50 units and compared with those in untreated serum rocked at the same temperature Each line represents one titration

was probably affected by the greater amount of G 50 but that C 2 was unaltered In the serum I C 3 was completely inactivated with the largest amount of G 50 and in the two other sera was definitely reduced in titre with both the G 50 amounts used

In a similar experiment two normal sera were treated at 4° C for 38 hours with the addition of EDTA No reduction in C 3 occurred

Thus it was found that C 2 was not affected by incubation with dextran gel at 4° C even after 38 hours As regards C 1 and C 4 no certain effect or a small reduction was noted In one of the sera C 3 had completely disappeared after 38 hours treatment but inactivation at 4° C as at 37° C was cation dependent Finally there was a tendency to an increased effect on C 3 with diminution in the degree of cross linking also at this low temperature

2) *Treatment at 16° C* In the treatment of serum with zymosan at 15 17 C properdin becomes bound to the zymosan As a result of such treatment about 20 per cent of all human sera lose almost all properdin (10) (14) and renewed treatment with zymosan at 37° C results therefore in no further C 3 inactivation since this requires the presence of properdin

The question whether the dextran gel behaved in a similar way to zymosan at 16° C was therefore studied In a series of experiments with the incubation of different individual sera or pooled sera with types G 25 and G 75 at 16° C it was found that subsequent treatment

with new dextran gels at 37° C led to a very extensive C'3 loss It was never possible to obtain a potent RP-reagent with respect to its content of C'3 In a few experiments with pooled serum C'3 was completely inactivated in the second treatment stage at 37° C

c) *Effect of Cations in the Treatment of Serum with Dextran Gel*

Inactivation of C'3 with zymosan requires Mg ions (8) The following experiments were designed to study whether or not dextran gel required cations to inactivate C'3 The *gel* was *washed* with *0 145 M NaCl* containing 0 1 M Na₃ EDTA, which EDTA concentration was also present in the serum to be treated

If the cations are bound by EDTA C'3 was not inactivated in a serum incubated with sufficient gel to inactivate fully all C'3 if cations are present Complement factors C'1 and C'4 are not affected (Fig 6) Even if the amount of dextran gel is doubled this is still true after 6 hours' treatment at 37° C In the presence of cations, when C'3 is completely inactivated, a marked reduction in C'1 and C'4 is already evident after 1hour's incubation (Fig 7) C'2 was not affected by 2 hours' treatment at 37° C in the presence of cations

The experiments thus showed that the inactivation of C'3 by dextran gels is cation dependent It is also clear that when C'3 is inactivated in the presence of cations, C'1 and C'4 are also reduced, but C'1 and C'4 are not affected if EDTA is present

f) *Effect of other Serum co Factors*

Pillemer et al (10) have shown that the PZ complex did not inactivate either C'3 in a serum, which had first been heated to 52 °C for 20 minutes, or C'3 in the complement reagents R1, R2 and R4 (10) As

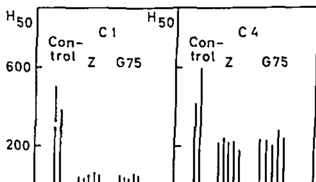


Fig 7

Treatment of serum with zymosan and dextran gel in the presence of Ca and Mg ions The effect on C'1 and C'4

C'1 and C'4 titres in serum treated with optimal amounts of zymosan and G 75 at 37° C for 60 minutes compared with titres in untreated serum controls Each line represents one titration

TABLE 3

Activity of C'3 after Incubation with Zymosan or Dextran gel G 50 at 37°C for 60 minutes in Serum Previously Heated at 52° C for 20 minutes and in Complement Reagents R1 R2 and R4

Treatment	C 3 titre in H50			
	Serum heated to 52 C for 20 minutes	R1	R2	R4
No treatment	57	26	160	80
37° C, 60 min	54	14	57	66
37° C 60 min with zymosan	44	8	20	57
37° C 60 min with dextran gel	50	9	26	60

may be seen in Table 3 C 3 in heated serum and in R4 is not affected by dextran gel treatment In R1 there is apparently a reduction in C 3 but this is not very certain due to the difficulty in estimating such low titre values The reduction of C 3 in R2 is very marked compared with that in untreated serum This reduction can perhaps be explained by the tendency of the euglobulins to flocculate when in contact with dextran gel thus rendering a nonspecific inactivation of C 3

The experiment thus shows that dextran gel like zymosan does not inactivate C 3 in a previously heated serum or in the complement reagent R4 and probably not in R1 and R2

g) *The Effect of Periodate Oxidation of the Dextran Gel on Human Serum Complement*

As previously shown (see Fig 3) the ability of dextran gel to inactivate *C 3* is inversely *proportional to the degree of cross linking* In other words the fewer cross linkages the more effective seems the gel This could mean that certain functional groups in the *glucose sub* units were responsible The degree of cross linking gives however no information on the relative proportion of for example 1–4 and 1–3 cross linkages By periodate oxidation information can be obtained concerning the number of unsubstituted glucose residues (1) During periodate oxidation a number of glucose *rings* are *broken* and it is highly possible that the steric configuration of the polysaccharide molecule is altered

A study of the effect on C 3 of dextran gels oxidized to different extents might then give information about which chemical groups are responsible or if *steric factors* are operative

Preliminary experiments showed that it required only one fifth more of a fully oxidized gel (type G 75) than of *unchanged G 75* to inactivate C'3 in the same amount of serum At the same time almost complete inactivation of the *other* complement factors occurred No anti-complementary action of the treated serum was found Periodate

with new dextran gels at 37° C led to a very extensive C'3 loss It was never possible to obtain a potent RP-reagent with respect to its content of C'3 In a few experiments with pooled serum C'3 was completely inactivated in the second treatment stage at 37° C

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f) *Effect of other Serum co-Factors*

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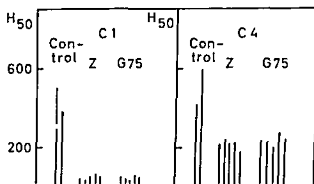


Fig 7

Treatment of serum with zymosan and dextran gel in the presence of Ca and Mg ions The effect on C'1 and C'4

C'1 and C'4 titres in serum treated with optimal amounts of zymosan and G 75 at 37° C for 60 minutes compared with titres in untreated serum controls Each line represents one titration

proximately 1 2 4) is however greater than can be accounted for by an increased available surface area in the more swollen gels Such a simple model of the swollen gel surface may however be quite erroneous and further causative factors must be sought such as may depend on the cross linking patterns It thus seems feasible that a gel with few cross linkages may have a greater number of functional C 3 inactivating groups on the grain surface

The mechanism underlying the ability of dextran gels to inactivate C 3 seems to have great similarities to that of zymosan since the same factors seemed important for both

The current views on the mechanism of the zymosan action are somewhat divergent As mentioned above *Pillemer et al* considered that C 3 is selectively inactivated by the PZ complex and that the other complement factors are not or hardly at all affected No attempt was made in the present work to isolate properdin from dextran gel incubated with fresh serum but it is possible that dextran gel (Dxg) and properdin form a complex which inactivates C 3 The results showed that when C 3 is inactivated with dextran gel at 37° C C 1 and C 4 are *also partially inactivated This was also true of the zymosan batches* used in these experiments These finding do not of course preclude that C 3 is inactivated by either a PZ or a P Dxg complex Since however dextran gel was so efficient an inhibitor that C 3 in all sera so far tested was inactivated while zymosan was only effective in 15 per cent of human sera (10) the greater potency of the dextran gel might mean that properdin is more effectively bound If such be the case the C 3 loss at 37° C in a serum first treated at 16° C should not be so large In pooled serum however C 3 could be inactivated completely in the second incubation stage at 37° C this should mean that the inactivation did not occur through the mediation of a P Dxg complex

Nelson (14) found that zymosan treatment parallel with C 3 also inactivated C 1 and C 4 He concluded that C 3 was inactivated through a PZC$_{1+4}$ complex but he did not however give any data on the behaviour of C 2 in the zymosan experiment

In the dextran gel induced inactivation of C 3 in serum C 2 was characteristically unaffected It seems therefore somewhat unlikely that as far as dextran gel is concerned C 3 is inactivated through a P Dxg C$_{1+4}$ complex

The heat labile and hydrazine sensitive serum factor which is required for the zymosan inactivation of C 3 was originally thought to be of complement nature (C 1 or C 2 and C 4) (10) Since however zymosan can inactivate C 3 in a serum in which C 1, C 2 and C 4 have been completely inactivated by strepokinase doubt has been cast on the identity of these as co factors in the C 3 inactivation (10)

Dextran gel was found to lack the ability to inactivate C 3 in previously heat treated serum or in complement reagents containing C 3 This does not necessarily mean however that they are co-factors

oxidized gel also inactivated C'3 in a serum to which Na_3EDTA had been added. The complement factors in serum were also inactivated at +4° C, but the oxidized gel had no effect on the complement components in reagents R2 and R4.

It can thus be stated that a fully oxidized G-75 was five times more potent than the original gel with respect to the inactivation of C'3. The effect on the other complement factors was also more marked than with the unoxidized gel. The properties of the gel were changed by oxidation in two respects: 1) complement factors were now inactivated at +4° C and 2) inactivation of C'3 was now not dependent on cations.

DISCUSSION

It was found that dextran gels had the property of inactivating C'3. This interaction was similar to the inactivation induced by zymosan with respect to the following factors: it increases with time, with amount of gel, with increasing temperature and is dependent on the presence of cations. Like zymosan, dextran gel did not inactivate C'3 in a previously heated serum or in the individual reagents R1, R2 and R4.

The effect on C'3 was inversely proportional to the grain size of the gel (Fig. 4). This may be interpreted as indicating that the active effect is confined predominantly to the surface of the grains. This experiment was done with type G 75 which excludes molecules with a molecular weight larger than about 40,000 (1). The possibility cannot be excluded that C'3 has a lower molecular weight than 40,000 and has such a shape that it can penetrate the G-75 grains and that also some inactivation can take place inside the particle. In view of the relationship found between grain size and activity of the gel it seems very probable that the surface effect is dominant. The distribution curves for different grain sizes were obtained from data kindly supplied by A/B Pharmacia and appeared to be essentially similar in form. If further the grain sizes used are assumed to be approximately regular in shape (spheres or cubes) there is a good correlation between relative surface area and inactivation of C'3.

In comparing the results obtained with the dextran gels G 25, G 50 and G 75 (Fig. 3) it must be taken into account that G 75 swells most and G-25 least. This means that if it were assumed that the swollen grains have relatively smooth surfaces their total surface area is greatest with G-75 and least with G 25 if equal weights of gels with similar size distributions are compared.

If for example the grains be assumed to be perfect cubes, the relative total areas in the three gels are: G 25 = 1, G 50 = 1.2 and G 75 = 1.32. If the grains are assumed to be spherical, which gives the minimal surface, the relative areas are: G 25 = 1, G 50 = 1.6 and G 75 = 2.1. The difference in C'3 inactivating effect between the three gels (ap-

proximately 1:2.4) is, however, greater than can be accounted for by an increased available surface area in the more swollen gels Such a simple model of the swollen gel surface may, however, be quite erroneous, and further causative factors must be sought such as may depend on the cross-linking patterns It thus seems feasible that a gel with few cross linkages may have a greater number of functional C'3 inactivating groups on the grain surface

The mechanism underlying the ability of dextran gels to inactivate C'3 seems to have great similarities to that of zymosan, since the same factors seemed important for both

The current views on the mechanism of the zymosan action are somewhat divergent As mentioned above *Pillemer et al* considered that C'3 is selectively inactivated by the PZ complex and that the other complement factors are not, or hardly at all, affected No attempt was made in the present work to isolate properdin from dextran gel incubated with fresh serum but it is possible that dextran gel (Dxg) and properdin form a complex which inactivates C'3 The results showed that when C'3 is inactivated with dextran gel at 37° C, C'1 and C'4 are also partially inactivated This was also true of the zymosan batches used in these experiments These finding do not, of course preclude that C'3 is inactivated by either a PZ or a P-Dxg complex Since, however, dextran gel was so efficient an inhibitor that C'3 in all sera so far tested was inactivated, while zymosan was only effective in 15 per cent of human sera (10), the greater potency of the dextran gel might mean that properdin is more effectively bound If such be the case the C'3 loss at 37° C in a serum first treated at 16° C should not be so large In pooled serum, however, C'3 could be inactivated completely in the second incubation stage at 37° C, this should mean that the inactivation did not occur through the mediation of a P-Dxg complex

Nelson (14) found that zymosan treatment, parallel with C'3 also inactivated C'1 and C'4 He concluded that C'3 was inactivated through a PZC'_{142} complex but he did not, however, give any data on the behaviour of C'2 in the zymosan experiment

In the dextran gel induced inactivation of C'3 in *serum*, C'2 was characteristically unaffected It seems therefore somewhat unlikely that, as far as dextran gel is concerned, C'3 is inactivated through a *P-Dxg C'_{142} complex*

The heat labile and hydrazine sensitive *serum factor* which is required for the zymosan inactivation of C'3 was originally thought to be of complement nature (C'1 or C'2 and C'4) *(10) Since*, however, zymosan can inactivate C'3 in a serum in which C'1, C'2 and C'4 have been completely inactivated by strepokinase, doubt has been cast on the identity of these as co-factors *in the C'3* inactivation (10)

Dextran gel was found to lack the ability to inactivate C'3 in previously heat treated serum or in *complement* reagents containing C'3 This does not necessarily mean, however, that they are co-factors

required for the inactivation of C′3 It is quite possible that other physical or physico-chemical events occurring in the heat treatment of serum, or in the preparation of complement reagents might lead to an increased resistance in some or all of the three C′3 subcomponents to the subsequent gel treatment From this it seems that the mechanism behind the ability of high molecular weight polysaccharides to inactivate C′3 remains obscure It cannot, in any case, be stated with certainty that the mechanism of dextran gel is similar to that postulated for zymosan

The action of periodate oxidized gel on complement were in several respects quite different from the action of unchanged gel Systematic studies with gels oxidized to different degrees should provide further information about the mechanism of this effect of high molecular weight polysaccharides on complement The results of such studies are to be published later on Finally the finding that dextran gel in contrast to zymosan, was able to inactivate C′3 in all human sera studied so far, makes it reasonable to assume that dextran gel can replace zymosan in the preparation of R3 reagents

SUMMARY

Human serum was treated with large molecular dextran gel (Sephadex®) and the effect on the complement factors was studied Particular attention was paid to the effect on the third component, C′3 The inactivation of this complement factor by dextran gel was, as with zymosan inactivation, dependent on the time, amount of gel, temperature, cations (Ca^{++}), (Mg^{++}) and certain co-factors in the serum

The effect of the dextran gel was related inversely to the degree of cross-linking, *i e* the fewer the cross-linkages, the larger the inactivating effect

C′3 was inactivated strongly even at 4° C with 17–38 hours's incubation, at the same incubation period C′1 and C′4 showed possibly a slight reduction while C′2 was unaffected At 37° C in the presence of divalent cations C′3 was completely inactivated, there was partial inactivation of C′1 and C′4 but there was no change in C′2

At 37° C in the presence of EDTA, dextran gel did not affect the complement factors even after incubation for 6 hours

In heat treated serum (52° C) and in the complement reagents R1, R2 and R4, C′3 was not obviously reduced after incubation for 60 minutes with dextran gel

Preliminary results of the effects of periodate oxidized dextran gel are given

The results are discussed with special regard to the underlying mechanism of the ability of high molecular weight polysaccharides to inactivate C′3

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THE BLOOD GROUPING AND THE BIOCHEMISTRY LABORATORIES OF THE STATE SERUM INSTITUTE HELSINKI, FINLAND

STUDIES ON COLIPHAGE T2R SPECIFIC ANTIGENS BY MEANS OF DOUBLE DIFFUSION PRECIPITATION AND IMMUNOELECTROPHORESIS

By

AITO LAHTI, JUSSI SAUKKONEN, TAPANI VAINIO and YRJÖ ALLAS

Received 13 III 63

The antigenic characteristics of coliphage T2r have been studied extensively. The main emphasis in these studies has been on the neutralizing antibodies. When intact phage was used for immunization, neutralizing antibodies appeared to be formed against the surface antigens of both the tail and the head (*Lanni & Lanni* 1957). The head antigens gave rise to antibodies which aggregated phage material and bound complement in the presence of intact phage particles. When disrupted phage was used for immunization several antibodies were formed against components originating in the interior of the phage. These antibodies were separated from those formed against surface antigens and seemed to be directed against internal proteins and, perhaps, DNA (*Levine et al* 1960). Only a rather limited number of reports deal with the precipitinogens of T2r. *Calis & Kaminski* (1959) found three antigenic components of T2r in immunoelectrophoresis. Two of these antigens moved towards the anode, one of them at a rate comparable to that of serum albumin. The third component migrated very little towards the cathode. A more recent report describes the presence of phage specific antigens by means of double diffusion precipitation, specifically demonstrating the presence of an antigenic component which is induced by the phage infection, but which is not found among the antigens of the completed phage particle (*Thomas & Suskind* 1960). In this investigation we have examined T2r specific antigens with the aid of double diffusion precipitation (DDP) and comparative immunoelectrophoresis (IE).

METHODS

Bacterial antigens. The preparation of E coli B antigens was described in the preceding paper (*Lahti et al* 1963).

Phage antigens. Coliphage T2r (obtained from *Dr Borek*, Columbia University, New York) was propagated in a conventional manner. E coli B was grown as described previously in 600 ml batches (*Lahti et al* 1963) to a density of about 2×10^{8} cells/ml

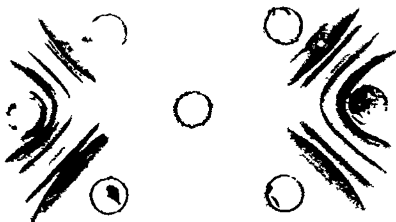


Fig 1
Precipitation arising from the reaction of E coli B and T2r antigen material with anti T2r serum in DDP The phage antigen is in the far right hand and far left hand reservoirs The bacterial antigen is in the center The two top and bottom reservoirs are filled with anti T2r serum

...luted phage preparations following the schedule given in the previous paper Complete Freunds adjuvant was used in the beginning of each month of immunization

The techniques for DDP and IE were described in the preceding paper

RESULTS

Anti T2r serum revealed 7 precipitation bands when it reacted with phage material in DDP (Fig 1) When this serum reacted with E coli B material only 2 rather faint bands where observed (Fig 1) A reaction of non identity was seen when anti T2r serum reacted with phage on one hand and with E coli B on the other This was shown in greater detail in Fig 2 It thus appears that our anti T2r serum contained several phage specific precipitins

When the T2r phage antigen preparation was fractionated electrophoretically and reacted with anti T2r serum (Fig 3) three groups of precipitation bands were seen The group which moved fastest



Fig 2
Test of identity for bands formed by anti T2r serum (at the bottom) and the E coli B antigens (upper left) and by anti T2r serum and T2r antigens (upper right)

towards the anode consisted of 3 bands which we have designated A–1, A–2 and A–3 Among these A–2 band is the strongest, whereas A–1 and A–3 are rather weak The second group which was detected without difficulty was seen close to the antigen reservoir It consisted of two bands The first one was a long band which crossed the A–2 and A–3 bands, and has been labeled C–1 It seemed to be branched, one arm extending far towards the anode and the second one remaining close to the starting point Between the C–1 band and the antibody reservoir there was a shorter, faint band, C–2, which did not move in the electric field

Upon closer examination, a third group of antigens could be detected in the area between the A and C groups It was composed to two bands, B–1 and B–2 *These precipitations were seen more clearly in* comparative IE with bacterial antigen in the upper longitudinal basin In the comparative IE (Fig 3) A 2 and C 1 gave a reaction of non identity with the bacterial material The bands A 1, A 3, B–2 and C 2 did not reach the precipitation lines formed between E coli B antigen and the anti-T2r serum, leaving the question of the identity of these precipitations unanswered B 1 gave a react on of at least partial identity with bacterial antigens

It was *established that in IE 6 rabbit antisera against T2r* gave the same patterns, as far as the "strong" T2r precipitinogens A 2, C 1 and C–2 were concerned B–2 was given by most of the antisera There was some variation in the ability of different rabbit antisera to reveal the "weaker" precipitinogens A 1, A 3 and B 1 However, two out of 6 antisera gave the above "complete" pattern

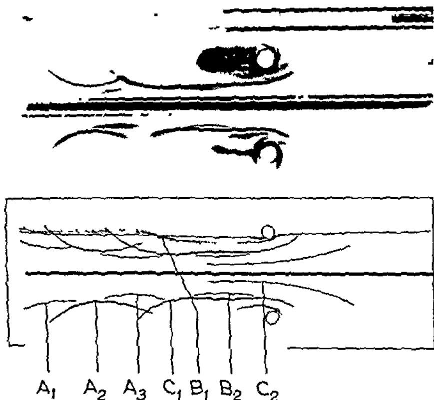


Fig 3

Comparative IE (the upper pattern) and ...
Phage material ...
serum (the lower ...

DISCUSSION

We have been able to *demonstrate* phage specific antigens with gel *diffusion* techniques. In the present investigation, special attention ...

The T2r specific precipitinogens shown here bear some resemblance to those reported by *Lalis & Kaminski* in T2r+ (1959). They demonstrated in IE a fast moving phage antigen which might correspond to our A antigens. The second antigen found by these authors hardly moved in the electric field thus behaving similar to our C-antigens. We were unable to detect an antigen which moved towards the cathode as



Fig 2
Test of identity for bands formed by anti-T2r serum (at the bottom) and the E coli B antigens (upper left) and by anti T2r serum and T2r antigens (upper right)

towards the anode consisted of 3 bands which we have designated A–1, A–2 and A–3 Among these A–2 band is the strongest, whereas A–1 and A–3 are rather weak The second group which was detected without difficulty was seen close to the antigen reservoir It consisted of two bands The first one was a long band which crossed the A 2 and A–3 bands, and has been labeled C–1 It seemed to be branched, one arm extending far towards the anode and the second one remaining close to the starting point Between the C–1 band and the antibody reservoir there was a shorter, faint band, C 2, which did not move in the electric field

Upon closer examination, a third group of antigens could be detected in the area between the A and C groups It was composed to two bands, B–1 and B–2 These precipitations were seen more clearly in comparative IE with bacterial antigen in the upper longitudinal basin In the comparative IE (Fig 3) A 2 and C 1 gave a reaction of non-identity with the bacterial material The bands A 1, A 3, B 2 and C 2 did not reach the precipitation lines formed between E coli B antigen and the anti-T2r serum, leaving the question of the identity of these precipitations unanswered B 1 gave a reaction of at least partial identity with bacterial antigens

It was established that in IE 6 rabbit antisera against T2r gave the same patterns, as far as the "strong" T2r precipitinogens A 2 C 1 and C–2 were concerned B 2 was given by most of the antisera There was some variation in the ability of different rabbit antisera to reveal the "weaker" precipitinogens A–1, A 3 and B 1 However, two out of 6 antisera gave the above "complete" pattern

DEPARTMENT OF PATHOLOGY, KAROLINSKA INSTITUTET, STOCKHOLM, SWEDEN (HEAD: PROFESSOR BO THORELL)

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By

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The morphologic picture of a tumour disease and the manner in which it progresses are determined by, among other things, the type of tissue from which the tumour has originated. As regards a virus-induced tumour, its type will depend on the organ on which the virus exerts its cytopathogenic effect—that is to say, the cytotropism of the virus. Within the group known as "avian leucosis viruses" the cytotropism can vary considerably, depending on the strain of virus and chick used. Some strains of virus such as RPL 12 (3, 6), Beard's erythroblastosis strain R and myeloblastosis strain A (4), produce several different tumour conditions in the White Leghorn.

The avian leucosis viruses used at this institution are an erythroleukaemia virus, obtained originally from *Engelbreth-Holm*, and a myeloid leukaemia virus obtained from *Beard* in 1957 (Myelo pool E). The erythroleukaemia virus has displayed an extremely limited cytotropism on our strain of White Leghorn, and produces only erythroleukaemia (7, 8). The myeloid virus has a broader spectrum and produces not only leukaemic disorders but also renal tumours in the strain of White Leghorn. This variation in the tumourogenic effect was found to depend on the age of the chick inoculated with the virus; among newly hatched chicks leukaemia was predominant, whereas in chicks inoculated at 2 weeks renal tumours were more common (13).

Further experimental studies on the pathogenic effect of myeloid leukaemia virus have shown that leukaemia in some chicks has an atypical course, characterized by severe anaemia and subleukaemic blood picture. Moreover, some of the inoculated chicks developed visceral lymphomatosis. In the investigation reported below an examination has been made of the incidence of various pathological conditions produced by the myeloid leukaemia virus. In addition, haematological and histopathological studies have been performed, with special emphasis on the subleukaemic form of the leukaemia.

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MATERIAL AND METHODS

Fowl strain.—White Leghorn chicks of a random bred strain (Edo) were used for the experiments. Throughout the period of more than 15 years during which this strain has been used at this laboratory there has been no case of spontaneous leukaemia in untreated controls. No attempts were made to isolate inoculated chicks from untreated controls except keeping them in separate cages.

Virus strain.—The virus used was of a myeloid strain obtained originally from Dr J. Beard, who has published details of its properties (1). This virus strain has been passaged on chicks continuously since 1959.

As has been shown by *Beard et al.* there is a close association between the content of myeloid leukaemia virus and the dephosphorylizing activity of plasma from the leukaemic fowl on adenosine triphosphate (10). In the present study the adenosine triphosphatase activity was examined by recording the kinetics of the variation of the hydrogen ion concentration in a solution containing a known amount of the triphosphate to which was added a known quantity of plasma from leukaemic chicks. The velocity of the change in pH followed with phenol red as the indicator is proportional to the amount of phosphate liberated. This method was used for selecting plasmas which in control titrations would produce leukaemia in 80–90 per cent of newly hatched chicks (14).

The plasmas containing the virus were obtained by intramedullary inoculation of 1–3 day old chicks with 0.2 ml plasma from leukaemic chicks. After 12–20 days the blood of the majority of the chicks indicated severe leukaemia. In the terminal stage, when according to haematocrit determinations the blood contained 30–40 per cent white cells, 0.5 ml of 0.5 per cent heparine solution (Vitrum) was injected intravenously. Five minutes later the blood was withdrawn through an incision in the jugular vein and centrifuged at 2 500 rpm for 15 min at 4° C. After pipetting off 0.5 ml plasma for determination of adenosine triphosphatase activity, the rest of the plasma was frozen to —70° C. Before use the thawed plasma was centrifuged as before. The chicks were inoculated intravenously with doses of 0.2 ml.

Haematological values.—The peripheral blood picture was followed in smears stained by the May-Grunwald-Giemsa method and by haematocrit determinations. These were performed in the usual way in heparinized capillary tubes which were centrifuged for 5 minutes in a haematocrit centrifuge at 10 000 rpm (International). The samples were drawn routinely from all inoculated chicks once a week and from the diseased chicks once or twice daily.

Histological preparation.—The marrow was removed from both femurs and tibias and specimens were taken of the liver, spleen, kidneys, lungs, bursa Fabricii and of different parts of tumours. Fixation was performed with Stieve's fluid (mercuric chloride, formalin and glacial acetic acid). The sections were stained with haematoxylin and eosin and by Giemsa's method. Marrow sections were stained also by the method of van Gieson and sections of the spleen by Hueck's procedure for iron pigment.

RESULTS

Frequency of Various Conditions Associated with Myeloid Leukaemia Virus

Chicks of 4 different age groups were inoculated intravenously with virus (Table 1). To judge from the adenosine triphosphatase values the virus activity of the plasma was the same in all groups. The chicks inoculated at 14 and 21 days were consistently followed up until they were 6 months of age. Of those receiving virus at 1–7 days only certain series were followed beyond 2 months.

The column headed "Subleukaemic anaemia" in Table 1 includes chicks with extremely low haematocrit values as seen in Table 2. Between the two types of leukaemia there are transitional forms but, as Fig. 1 shows, these are few in number. It would therefore seem

TABLE 1

Frequency and Response Times of Various Conditions Associated with Myeloid Leukaemic Virus

Age at inoc (days)	No of chicks (use*)	Classic myeloid leukaemia		Subleukaemic anaemia		Visceral lymphomatosis		Kidney tumours		
		Per cent	Survival period (days)	Per cent	Survival period (days)	Incidence†	Survival period (days)	Incidence†	Response time (days§)	
1–3	135	74	M 18 (12–48)	4	M 24 (16–31)	3/7	M 149 (141–157)	1/7	105	
4–7	49	49	M 21 (15–74)	14	M 31 (19–57)	0/15		1/15	97	
14	51	33	M 26 (14–66)	18	M 22 (15–30)	3/16	M 149 (119–166)	2/16	M 90 (67–113)	
21	18	12	M 26 (17–36)	12	M 38 (12–63)	5/13	M 153 (107–205)	5/13	M 136 (63–205)	One animal died from haemangiomatosis 63 days after inoculation

* corrected for intercurrent deaths
† number diseased/number observed up to 6 months
§ response time from inoculation until clinically manifest kidney tumour

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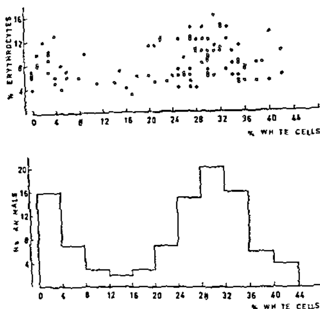


Fig 1

Haematocrit values of leukaemic chicks. Percentage of erythrocytes and granulocytes (upper graph) and the frequency distribution with respect to the percentage of white cells (lower graph)

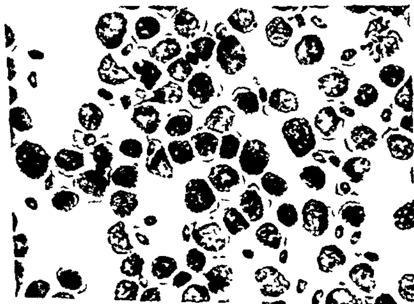


Fig 2

Typical blood smear from a chick with the classic type of myeloid leukaemia (*May Grünwald Giemsa* × 9[illegible])

justified to divide the leukaemia material into 2 main groups, the larger one containing chicks dying of classic myeloid leukaemia and the other group chicks with severe anaemia and leukopenia No exact boundary line can be drawn between the two groups and the classification as subleukaemic anaemia is therefore approximate

As Table 1 shows, the frequency of classic leukaemia was highest in the youngest age groups, with 74 per cent takes, it decreased with age down to 12 per cent for the groups inoculated at 21 days Two-month-old chicks, which had been inoculated with large doses of virus, were all resistant (unpublished data)

The time elapsing between inoculation and death (survival period) varied widely within the various groups It tended to be slightly shorter in the youngest groups, but there was obviously no essential difference between the groups in this respect The first deaths occurred 12 days after inoculation, and the mean survival period for the various groups ranged from 18 to 29 days

Subleukaemic anaemia was uncommon among chicks inoculated at 1–3 days In the higher age groups, on the other hand, it was more frequent, being found in 12–18 per cent of the inoculated chicks The percentage of subleukaemic anaemia, calculated per total number of leukaemic chicks, was gradually increasing, from 5 per cent in the lowest age group and 22 and 35 per cent in the groups inoculated at 4–7 and 14 days respectively, to 50 per cent in the highest age group There was no significant difference between the survival periods for the two forms of the disease

The survival period was considerably longer for visceral lymphomatosis and renal tumours than for leukaemia, ranging from 107 to 205 days and 63 to 205 days, respectively The relatively small recorded number with these diseases can be explained partly by the great number of chicks dying in myeloid leukaemia leaving only a limited number alive during the period when lymphomatosis and renal tumours do develop The figures in the different age groups are too small to allow any inter-group comparison

One of the chicks inoculated at 21 days had haemangiomatosis and death occurred 63 days after inoculation

One of the chicks inoculated at 21 days and sacrificed at 84 days when moribund through atypical leukaemia had also a tumour in one kidney, and one chick which was inoculated at 21 days and died from lymphomatosis in 158 days had a small renal tumour Apart from these cases only one of the various pathological conditions was found in any particular chick

Histologic Findings

Classic leukaemia

In the terminal stage all the chicks had severe peripheral leukaemia, with excessive haematocrit values for the white cells (Fig 1), corre-

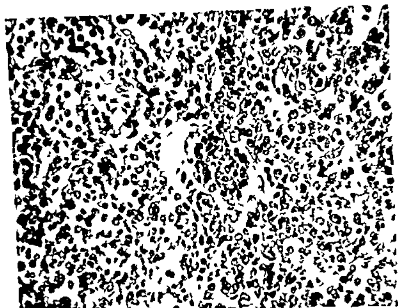


Fig 5

Section of lung in a case of classic leukaemia at the terminal stage *Giemsa* × 400

has pseudopodia like processes The cytoplasm is rather scanty, moderately basophil and often has a small perinuclear halo, with a fine vacuolisation and sometimes a delicate granulation can be seen (Fig 2)

Most of the nuclei are round but oval or slightly crescent or dumbell shaped ones are occasionally seen The finely trabecular chromatin often has irregularly condensed patches and fairly prominent nucleoles

The leukaemia cells differ distinctly from the other cells in the peripheral blood Only lymphatic cells display a certain similarity to them The coarse chromatin pattern in the lymphocyte nucleus however, is characteristic

There is no clear cytologic difference between leukaemia cells in the acute and more protracted forms of the disease In both cases the condition is myeloblastic leukaemia and there is no type of chronic myelosis with cell maturation as is seen in man

Subleukaemic Anaemia

The prominent features of the subleukaemic anaemia distinguishing it from the classic form of the leukaemia was a pancytopenia appearing in both early and late stages of the leukaemic disease Two cases that exemplify the course are shown in Fig 6

Data on the chicks with subleukaemic anaemia are surveyed in Table 2



Fig 3 Fig 4

Severe leukaemic infiltration of spleen and liver, respectively, in cases of classic leukaemia at the terminal stage *Haematoxylin and eosin* × 110

sponding to 1–1 5 million leukaemia cells per cu mm (9) The number of erythrocytes was extremely low (Fig 2)

The bone marrow, the principal organ involved in this tumourous disease, was grey and somewhat gelatinous The sections showed a carpet of monomorphous leukaemia cells, which obliterated the normal architecture The femur marrow was devoid of fat but small amounts were present in the tibia Normal haematopoiesis was almost completely absent

Among the other organs the spleen, liver, lungs, kidneys and bursa Fabricii were examined The first three of these displayed advanced leukaemic infiltration in the terminal stage of the disease The spleen was greatly enlarged and only parts of Malpighian bodies remained, while the red pulp was completely leukaemic (Fig 3) The liver, too, was enlarged, and extensive confluent periportal infiltrates were a distinctive feature of the pathologic picture In the interjacent liver parenchyma fairly sparse disseminated infiltration was noted (Fig 4)

The alveolar walls of the lungs, which were reddish grey and athelectatic, displayed a compact leukaemic infiltration (Fig 5)

The kidney showed less advanced changes They were slightly enlarged and there was focal infiltration of leukaemia cells but no appreciable reduction of the parenchyma

The bursa Fabricii, the lymphoid organ at the cloaca as a rule showed no tumour cell infiltration in the myeloid leukaemia

Cytologic picture of the leukaemia cells —The leukaemic cell flora consists of immature atypical cells from the myeloid series They are round, with diameters between 10 and 15 microns and the surface often



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TABLE 2

Haematocrit and Histopathological Features of Animals with Subleukaemic Anaemia

Animal no	Age at inoc days	Age at death days	Haematocrit		Autopsy findings						
					Leukaemic infiltration in*					Bone marrow necrosis	Spleen siderosis
			R	W	Bone marrow	Spleen	Liver	Kidneys	Lungs		
1	1	17	5	4	++++	+++	+++	++	+++	—	—
2	1	18	7	3	++++	+++	++++	+++	+++	—	—
3	1	25	8	1	++++	++	+	++	++	—	—
4	1	30	6	8	++++	++++	+++	+++	+++	—	—
5	1	14	6	4	++++	++++	++++	+++	+++	—	+
6	3	19	8	5	++++	+++	+++	+++	+++	—	—
7	5	24	12	2	+++	+++	++	++	++	—	+
8	5	33	9	1	+++	++	++	++	+++	—	+++
9	5	33	10	3	++++	++	+++	++	++	—	+
10	7	30	6	6	++++	+++	+++	++	+++	—	+
11	7	30	7		++++	++	++	++	++	—	++
12	14	32	6		++	++	+	+	—	+	+++
13	14	35	7	1	++++	++	++	++	+++	—	—
14	14	36	4	1	++	++	+	+	+	—	—
15	14	39	4		++++	+++	+++	++	++	+	—
16	14	29	9	3	++	++	+	++	++	+	—
17	14	23	8	1	++	++	+	++	+	+	+++
18	14	33	6		+++	++	++		+++	++	+
19	14	44	4	5	++++	+++	++++	++	+++	—	+
20	14	44	7	6	++++	+++	++++	++	++	—	—
21	21	33	7		+	—	++†	+	—	+	++
22	21	60	10	1	++	++	+	—§	—	+	+

* + occasional leukaemia cells
++ several leukaemia cells often in small foci
+++ marked leukaemic infiltration with several large foci
++++ heavy leukaemic infiltration
The grading of bone marrow necrosis and spleen siderosis has been done in a similar way

† Microscopic examination revealed small necroses in the liver of this chicken
§ One tumor of adenosarcomatous type in the left kidney

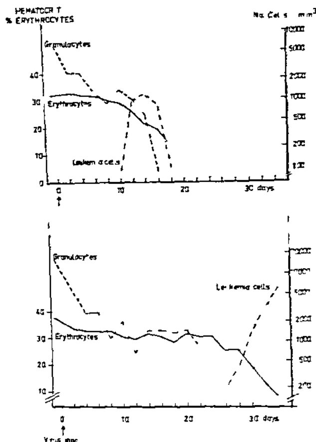


Fig 6
Haematological course for two chicks with subleukaemic anaemia.

The histological picture of the organs from some of these chicks did not differ appreciably from that in ordinary leukaemia (*e g* chick nos 5, 6 and 19) In many cases there was massive leukaemic infiltration of the marrow but the peripheral infiltration was comparatively mild (*e g* nos 3 and 11) In some cases the infiltration of the marrow in the terminal stage of the disease was scant and diffuse, and the fat content was normal (*e g* nos 12, 14, 16, 17, 21 and 22) Besides, in these chicks there was no prominent infiltration of the other organs A prominent histological feature of these marrows was lymphoid hyperplasia, sometimes diffuse and sometimes in the form of follicles, the centres of which were occupied by immature nucleolated cells It was not possible to determine the precise nature of these cells from the histological picture, they may have been myeloid leukaemia cells or they may have been lymphoblasts and thus correspond to germ centres (Fig 7)

Another distinguishing and conspicuous feature of the marrow in

TABLE 2

Haematocrit and Histopathological Features of Animals with Subleukaemic Anaemia

Animal no	Age at inoc days	Age at death days	Haematocrit R	Haematocrit W	Autopsy findings: Leukaemic infiltration in*) Bone marrow	Spleen	Liver	Kidneys	Lungs	Bone marrow necrosis	Spleen siderosis
1	1	17	5	4	++++	+++	+++	++	+++	—	—
2	1	18	7	3	++++	+++	++++	+++	+++	—	—
3	1	25	8	1	++++	++	+	++	++	—	—
4	1	30	6	8	++++	++++	+++	+++	+++	—	—
5	1	14	6	4	++++	++++	++++	+++	+++	—	+
6	3	19	8	5	++++	+++	+++	+++	+++	—	—
7	5	24	12	2	+++	+++	++	++	++	—	+
8	5	33	9	1	+++	++	++	++	+++	—	+++
9	5	33	10	3	++++	++	+++	++	++	—	+
10	7	30	6	6	++++	+++	+++	++	+++	—	+
11	7	30	7		++++	++	++	++	++	—	++
12	14	32	6		++	++	+	+	—	+	+++
13	14	35	7	1	++++	++	++	++	+++	—	—
14	14	36	4	1	++	++	+	+	+	—	—
15	14	39	4		++++	+++	+++	++	++	+	—
16	14	29	9	3	++	++	+	++	++	+	—
17	14	29	8	1	++	++	+	++	+	+	+++
18	14	33	6		+++	++	++		+++	++	+
19	14	44	4	5	++++	+++	++++	++	+++	—	+
20	14	44	7	6	++++	+++	++++	++	++	—	—
21	21	33	7		+	—	++†	+	—	+	++
22	21	60	10	1	++	++	+	—§	—	+	+

* + occasional leukaemia cells
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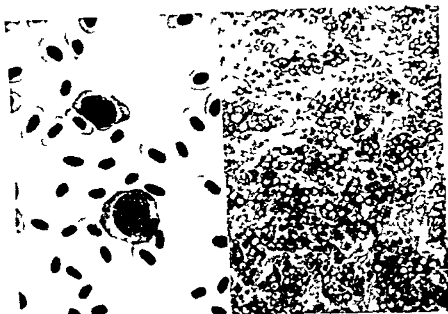


Fig 9 Fig 10

Fig 9 Visceral lymphomatosis atypical lymphoid cells in a blood smear Giemsa × 700

Fig 10 Visceral lymhomatosis section of spleen showing the periphery of a tumour nodule (below) and the scattered tumour infiltration (above) Giemsa × 320

tissue between such foci also displays such infiltration but of mild degree Tumour cells may also be seen in the peripheral blood though only to a small extent (Fig 9)

A regular feature in marrow displaying lymphomatous infiltration is focal sclerosis with less marked tumour infiltration than in the surrounding tissue (Fig 11)

Kidney Tumours

The renal tumours ranged in size from 0 2 to about 5 cm in diameter The free surface of the tumour was covered by a thin capsule Grossly the tumours were clearly demarcated from the residual tissue and readily dissected but there was no actual encapsulation here The consistency varied from firm to soft in places myxomatous both from one tumour to another and within a particular tumour The cut surface was greyish white often with small cysts containing a clear yellow fluid Small haemorrhages were common and in some cases there were haematomas some of which ruptured the capsule with bleeding into the abdominal cavity as a consequence In none of the chicks was neoplastic tissue seen to penetrate the capsule involving spread of the

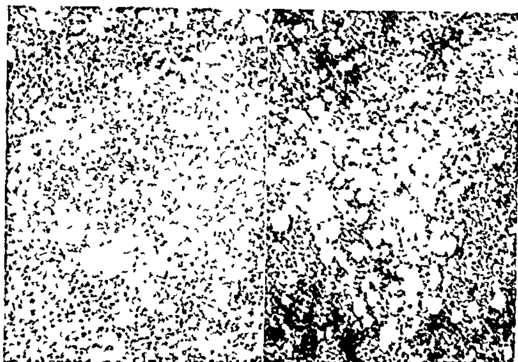


Fig 7 Local lymphoid hyperplasia in the marrow of a chick with subleukaemic anaemia *Giemsa* × 400
Fig 8 Recent marrow necrosis in a chick with subleukaemic anaemia *Giemsa* × 110

subleukaemic anaemia was the presence of small necroses (Fig 8) The devitalized tissue could not be identified and it was therefore impossible to establish which cells had undergone necrosis, especially as in this form of leukaemia the infiltration of the haematopoietic tissue by leukaemia cells was diffuse The necrosis, however, seemed to involve both leukaemia cells and normal haematopoietic cells

Other fairly regular features were splenomegaly and splenic siderosis In some cases the iron deposits were small but in others considerable No such deposits were observed in normal chicks or those dying from classic myeloid leukaemia

Visceral Lymphomatosis

The histological features of this disease have been thoroughly described by *Burmester et al* (6) It may suffice here to touch on a few relevant details

Visceral lymphomatosis constitutes a neoplasm involving lymphatic tissue, and the atypical, moderately polymorphous tumour cells are of lymphatic type (Fig 9) In the terminal stage the disease presents a multifocal picture, a characteristic feature is the presence in the organs of well defined foci, massively infiltrated by tumour cells (Fig 10) The

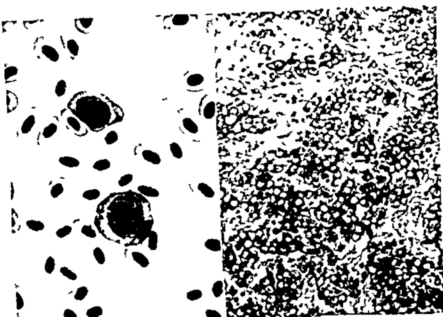


Fig 9 *Fig 10*

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Fig 10 Visceral lymhomatosis section of spleen showing the periphery of a tumour nodule (below) and the scattered tumour infiltration (above) *Giemsa* × 320

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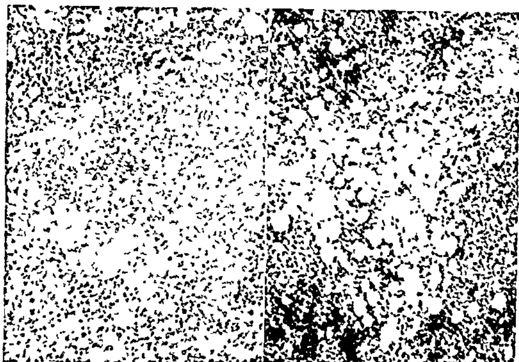


Fig 7 Fig 8

Fig 7 Local lymphoid hyperplasia in the marrow of a chick with subleukaemic anaemia *Giemsa* × 400
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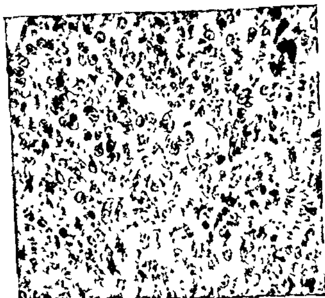


Fig 13
Renal tumour with sarcomatous structure of the fibromatous component *Haematoxylin and Eosin* × 400

tumour cells into the abdominal cavity No distant metastases were noted but in some cases there were multiple tumours in both kidneys

The histological picture of the tumours accords closely with that reported by *Thorell* (13) They were of the mixed type, with both epithelial and mesenchymal components, both varying considerably in their degree of differentiation in a particular tumour As a rule, however, the epithelial component was highly differentiated and developed adenomatous formations and also epithelial pearls The mesenchymal component gave rise to fibromatous and myxomatous tissue, cartilage and bone (Fig 12) Where the differentiation was less advanced, there was a predominance of fibromatous tissue having a sarcomatous structure, with nuclear polymorphism and numerous mitotic figures (Fig 13) In spite of this low degree of differentiation, these parts of the tumours displayed no marked tendency to infiltration and were clearly circumscribed There was no actual encapsulation, however

DISCUSSION

As shown in Table 1, the incidence of classic myeloid leukaemia decreased with age, from 74 per cent at 1–3 days of age at inoculation to 12 per cent at 21 days This is in close agreement with earlier findings by *Thorell* (13) As regards the subleukaemic anaemia, there were few takes in the lowest age groups, ranging from 12–18 per cent However,

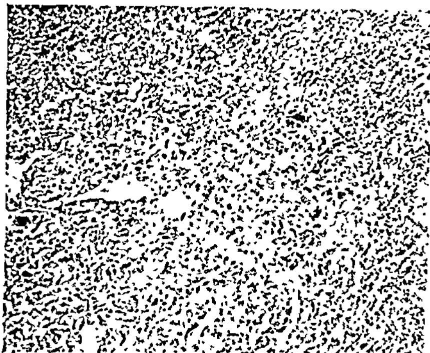


Fig 11

Visceral lymphomatosis focal marrow sclerosis with sparse tumour cell infiltration (*below*) compared with the more severe infiltration in the non-sclerotic marrow (*above*) *Haematoxylin and Eosin* × 200

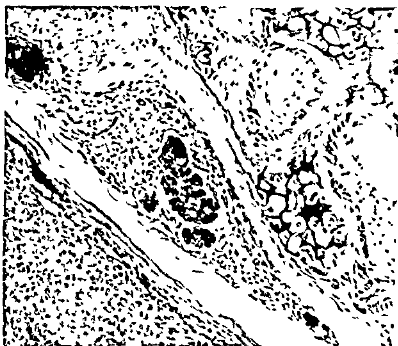


Fig 12

Section of a renal tumour showing an epithelial component (*centre*) osseous and chondromatous components (*right*) and a fibromatous area (*bottom*) *Haematoxylin and Eosin* × 140

distant metastases would suggest a multifocal attack of the virus, although the possibility of a local invasion by tumour cells cannot be ruled out.

The absence of distant metastases in spite of the sarcoma-like structure seen in several of the tumours may be due to the fact that the chicks were sacrificed as soon as a clinical diagnosis of renal tumour was made. In *Thorell's* study, where the chicks were killed in the terminal stage, cases of distant metastases of kidney tumours were found (13).

The division of the leukaemic chicks into two groups, one of classic myeloid leukaemia and the other of subleukaemic anaemia was approximate and performed in order to examine whether the two courses of the disease could be distinguished histologically.

Certain features would seem to be distinctive of the subleukaemic anaemia. The peripheral leukaemic infiltration was often less marked than in classic myeloid leukaemia and in several of the cases the marrow was also less extensively involved, even though the chicks were not sacrificed until the terminal stage of the disease. Several of these marrows without massive leukaemic changes displayed marked lymphoid hyperplasia and small areas of recent necrosis. Obviously the leukaemic proliferation in these cases did not progress to its full extent (Fig. 6).

The erythrocyte concentration was reduced both in the classic leukaemia and the subleukaemic anaemia but considerably more so in the latter in relation to the leukaemic proliferation in the marrow. It would therefore seem to have some other cause than neoplastic replacement of the marrow. The splenic siderosis indicates destruction of the erythrocytes.

The genesis and nature of the subleukaemic anaemia remain obscure. It is possible that the low grade of the leukaemic infiltration partly depends on a lower proliferative activity of the leukaemic cells than in the classic form of the disease (2). A direct cytotoxic effect of the virus on the bone marrow cells and the erythrocytes might also be responsible for the atypical course of the leukaemia. It is of special interest, however, that the virus in use induces neoplastic transformation of tissue with antibody forming capacity (5). A possible autoimmune reaction as the cause of subleukaemic anaemia is the subject of further investigations.

SUMMARY

A study has been made of the pathogenic effect of myeloid leukaemia virus on the chick. As a result of inoculation with the virus [illegible] samples most of [illegible]
classic form [illegible]
leukaemia follow[illegible]
kaemic anaemia [illegible] of which were severe anaemia

the percentage of subleukaemic anaemia, calculated per total number of leukaemic chicks was gradually increasing from 5 per cent in the lowest age group to 50 per cent in the highest age group

Of the animals escaping the leukaemia and being observed for up to 6 months many developed visceral lymphomatosis and kidney tumours, which demonstrate the broad pathogenic spectrum of the virus The pathological-anatomical picture of the diseases are almost identical with those found by *Burmester et al* in their large experimental series (4), but in the present study no osteopetrosis was detected, and some of the leukaemic chicks were found to display an atypical course of the disease

It is still uncertain whether the virus preparations which we have used in this series of experiments contain only one pure virus strain having several different target organs, or whether there is a combination of several distinct viruses each having its own target organ As has also been shown earlier by *Thorell* (13), the frequency of takes is dependent on the age of the chick at inoculation and also on the amount of virus injected The higher susceptibility to the virus of the new-hatched chicks might be due to these chicks being less immunologically competent than the older ones and thus unable to evoke an efficient antibody response to the virus infection

The series of *Burmester et al* contained several cases of visceral lymphomatosis among the controls Over the last 4 years 30–60 fowls at this laboratory have been in contact with inoculated chicks for 12 months, but no case of lymphomatosis has been encountered among the noninoculated fowls However, since lymphomatosis does occur in the strain the disease would certainly have appeared among the controls had they been observed over a longer period

The discovery of lymphomatosis in several chicks within a fairly short period of the inoculation indicates that the disease was produced by contamination with the virus It may then be a result of a direct cytopathogenic effect, with the inoculated virus attacking both the myeloid and the lymphatic cells—that is to say, the virus preparation would contain 2 viruses, or one virus with 2 target organs It is also possible that there was an indirect effect, whereby the lymphomatosis is present in the fowl strain but remains latent owing to formation of antibodies against the virus (11) When the myeloid virus is administered it affects antibody-producing tissue (5), and might thus interfere with the resistance against the lymphomatosis virus

The kidney tumours that appeared in this series were all of a mixed type with both mesenchymal and epithelial components From the histogenetic aspect they appear to be of the teratoid type, with their derivation in multipotent cells in the growing kidney The regular occurrence of such tumours in the inoculated chicks strongly indicates a viral aetiology, especially since these tumours very seldom appear spontaneously (12) The presence of multiple renal tumours with no

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THYROXIN-INDUCED CHANGES IN CELL COMPOSITION OF LYMPH NODE TISSUE, SPLEEN AND THYMUS

A Quantitative Examination on Young Guinea Pigs

By

ULF ERNSTROM

Received 31.i.63

It has been demonstrated that small doses of exogenous thyroxin cause an increase in the weight of the lymphatic tissue in guinea pigs (*Gyllensten* 1953). The histologic picture of such hyperplasia was studied by Ernstrom and Gyllensten in 1959. They observed that an increase of the pyroninophilic cells in the lymph nodes was the most conspicuous of all the relative changes affecting the cell composition of the lymph nodes, spleen and thymus, being very distinct only 3 days after the injection of thyroxin. In the present investigation, an analysis will be given of the early changes produced by thyroxin in the cell composition of the lymph node tissue, the spleen and the thymus. As the reaction to thyroxin in various lymph nodes seems contingent to the original composition of the cells, also the cytological differences between the individual groups of lymph nodes will be described.

MATERIAL AND METHODS

he & Co) per
the animal

All the animals were killed [illegible]
possible any differences due [illegible]
thyroid activity (*Fortuyn* [illegible] 1955) the animals
were sacrificed at the same time of day [illegible]

of haemolytic type, granulocytopenia and subleukaemic blood picture The leukaemic infiltration of the organs was often less marked for these chicks than for those with the classic form of the disease Distinctive histo pathological features of the subleukaemic anaemia were lymphoid hyperplasia, small necrotic areas in the marrow, splenomegaly and splenic siderosis

Visceral lymphomatosis, mixed tumours of the teratoid type in the kidneys, and one case of haemangiomatosis were recorded among chicks inoculated at 14 and 21 days

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TABLE 2

Comparison between the Cell Populations of the Cortical Layers of Cervical Scapular and Inguinal Lymph Nodes in Guinea Pigs Mean ± Standard Deviation

Lymph nodes	Number of animals	Reticular cells %	Lymphocytes %	Total pyroninophilic cells %	Plasma cell index
Cervical lymph nodes	19	18.8 ± 4.7	75.5 ± 5.1	5.6 ± 2.3	0.12 ± 0.11
Scapular lymph nodes	18	21.9 ± 5.0	74.6 ± 5.7	3.6 ± 2.0	0.15 ± 0.27
Inguinal lymph nodes	19	23.9 ± 5.8	68.6 ± 12.9	4.4 ± 2.4	0.07 ± 0.10

In the *spleen* of normal guinea pigs, the white pulp contains, relatively speaking, more lymphocytes than the red pulp and less reticular cells (Table 3). Apart from the germinal centres seen in the white pulp, most pyroninophilic cells are found in the perifollicular zone, surrounding the white pulp. This area includes cells that correspond morphological to the transitional cells and immature and mature plasma cells in the medulla of the lymph nodes. In the red pulp of the spleen, however, the pyroninophilic cells are less frequent than in the medulla of the lymph nodes.

TABLE 3

Comparison between the Cell Populations of the Red and White Pulp of the Spleen in Guinea Pigs Mean ± Standard Deviation

Part of spleen	Number of animals	Reticular cells %	Lymphocytes %	Pyroninophilic cells %	Plasma cell index
Red pulp	18	62.5 ± 8.1	30.1 ± 8.8	7.4 ± 4.6	0.19 ± 0.17
White pulp	18	31.1 ± 8.7	59.7 ± 10.1	9.5 ± 6.1	0.0

The medulla and cortex of the *thymus* in normal guinea pigs do not only differ from each other in density of cells, but also in relative cell composition. Thus, the cortical layers contain more lymphocytes and pyroninophilic cells and the medullary part relatively more reticular cells (Table 4). The difference in contents of reticular cells and lymphocytes is highly significant ($p < 0.001$), and in contents of pyroninophilic cells significant ($p < 0.01$). No plasma cells, nor germinal centres have been seen.

TABLE 4

Comparison between the Cell Populations of the Medullary and Cortical Parts of th Thymus in Guinea Pigs Mean ± Standard Deviation

Part of thymus	Number of animals	Reticular cells %	Pyroninophilic cells %	Lymphocytes %
Medulla	17	23.8 ± 2.9	4.4 ± 1.8	72.0 ± 3.5
Cortex	17	9.2 ± 2.7	6.6 ± 2.5	84.2 ± 4.1

the red and white splenic pulp were counted separately. The perifollicular zone of the spleen was ascribed to the red pulp. The same classification system was used with regard to the cells as adopted by *Fagraeus* (1948) and by *Ernström & Gyllensten* (1959). Reticular cells, lymphocytes and pyroninophilic cell types (transitional, immature and mature plasma cells) were distinguished. The group of lymphocytes includes small and medium sized ones, but not the so called large lymphocyte which is strongly pyroninophilic. In using these terms it must be borne in mind that not all transitional cells develop into plasma cells. However, at least in the thymus and cortical layers of the lymph nodes, where there are few or no plasma cells, the transitional cells may develop into medium sized and small lymphocytes. A transformation also in the opposite direction cannot be excluded.

The differential cell counts did not comprise the reaction centres in the cortical layers of the lymph nodes. These secondary follicles were instead analysed as to their contents of dividing cells. The number of mitoses in at least 4000 cells in the secondary follicles of the cervical lymph nodes of each animal was counted. The mitotic index was determined as percentage of dividing cells in the total cell number.

Apart from the cell types mentioned above, the sparse occurrence of a few others such as the neutrophilic and eosinophilic polynuclear cells was observed. The endothelial cells, muscle cells and fibroblasts in connection with the vessels were disregarded in the present investigation.

The results were statistically analysed by means of the Student's t series method.

RESULTS

Normal Animals

The various *lymph nodes* from normal animals differ from each other in appearance and cell composition. In the cervical lymph nodes of young guinea pigs the medullary parts are very conspicuous, while in the extremity nodes the cortical layers predominate. The contents of *pyroninophilic cells are larger in the cervical lymph nodes than in the* scapular and inguinal ones. Also the relative amount of plasma cells is greatest in the cervical nodes. By way of illustration, a comparison may be made between the plasma cell indices in the two node types (*i.e.* the ratio of immature and mature plasma cells to the total of pyroninophilic cells). These differences in cell composition are apparent in the medullary as well as in the cortical parts of the lymph nodes, being demonstrated in Tables 1 and 2, which refer to 19 young normal guinea pigs of both sexes. Despite the marked variation between individual animals, the aforementioned difference in the plasma cell index of the cervical and extremity lymph nodes is statistically significant ($p < 0.01$) and the difference in the contents of pyroninophilic cells highly significant ($p < 0.001$).

TABLE 1

Comparison between the Cell Populations of the Medullary Cords of Cervical, Scapular and Inguinal Lymph Nodes in Guinea Pigs. Mean ± standard Deviation

Lymph nodes	Number of animals	Reticular cells %	Lymphocytes %	Total pyroninophilic cells %	Plasma cell index
Cervical lymph nodes	19	43.1 ± 9.1	37.0 ± 11.7	20.0 ± 9.3	0.41 ± 0.19
Scapular lymph nodes	18	61.4 ± 9.7	31.1 ± 10.1	7.5 ± 5.9	0.23 ± 0.20
Inguinal lymph nodes	19	54.1 ± 12.3	34.7 ± 12.7	10.4 ± 8.6	0.22 ± 0.14

TABLF 2

Comparison between the Cell Populations of the Cortical Layers of Cervical Scapular and Inguinal Lymph Nodes in Guinea Pigs Mean ± Standard Deviation

Lymph nodes	Number of animals	Reticular cells *,	Lymphocytes *,	Total pyroninophilic cells *,	Plasma cell index
Cervical lymph nodes	19	18 8 ± 4 7	75 5 ± 5 1	5 6 ± 2 3	0 12 ± 0 11
Scapular lymph nodes	18	21 9 ± 5 0	74 6 ± 5 7	3 6 ± 2 0	0 15 ± 0 27
Inguinal lymph nodes	19	23 9 ± 5 8	68 6 ± 12 9	4 4 ± 2 4	0 07 ± 0 10

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Part of spleen	Number of animals	Reticular cells *,	Lymphocytes *,	Pyroninophilic cells *,	Plasma cell index
Red pulp	18	62 5 ± 8 1	30 1 ± 8 8	7 4 ± 4 6	0 19 ± 0 17
White pulp	18	31 1 + 8 7	59 7 ± 10 1	9 5 ± 6 1	0 0

The medulla and cortex of the *thymus* in normal guinea pigs do not only differ from each other in density of cells, but also in relative cell composition Thus, the cortical layers contain more lymphocytes and pyroninophilic cells and the medullary part relatively more reticular cells (Table 4) The difference in contents of reticular cells and lymphocytes is highly significant ($p < 0\ 001$), and in contents of pyroninophilic cells significant ($p < 0\ 01$) No plasma cells, nor germinal centres have been seen

TABLF 4

Comparison between the Cell Populations of the Medullary and Cortical Parts of the Thymus in Guinea Pigs Mean ± Standard Deviation

Part of thymus	Number of animals	Reticular cells *,	Pyroninophilic cells *,	Lymphocytes *,
Medulla	17	23 8 ± 2 9	4 4 ± 1 8	72 0 ± 3 5
Cortex	17	9 2 ± 2 7	6 6 ± 2 5	84 2 ± 4 1

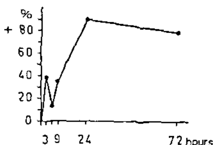


Fig 1
Percentage changes of plasma cell index in the medullary cords of cervical lymph nodes in guinea pigs after treatment with thyroxin

Thyroxin-treated Animals

Lymph nodes In the *medullary* cords the first change to be noticed after thyroxin treatment was the increase in the plasma cell index In the cervical nodes this was pronounced as soon as 3 hours after the administration of the hormone (Fig 1) and still more accentuated 24–72 hours after ($p = 0.01$) The scapular and inguinal lymph nodes were examined 24–72 hours after thyroxin administration In the inguinal lymph nodes the mean increase was almost as large as in the cervical ones (+ 63 per cent and + 94 per cent), but less in the scapular ones, being apparent only 72 hours after (+ 0 per cent and + 57 per cent) The quantity of plasma cells ascertained in the extremity nodes was small, and the determinations of the plasma cell indices therefore uncertain Accordingly, the statistical analysis revealed that the big changes of the means were not significant ($p < 0.2$)

There was a rise in the total number of pyroninophilic cells in the cervical lymph nodes 9 hours after the injection of thyroxin (Fig 2) This change was accentuated 24–72 hours after ($p < 0.05$) At the same time, in the extremity nodes there was no addition to the number of pyroninophilic cells The ratio between the lymphocytes and the reticular cells in the cervical lymph nodes was slightly higher 3, 6 and 9 hours after injection (+ 7, + 2, + 3 per cent respectively) and somewhat lower 24–72 hours (—19, —2 per cent, respectively) after the administration of the hormone, but these changes were not significant

In the *cortical layers* of the cervical lymph nodes the plasma cell index had risen 3 and 6 hours after the injection of thyroxin However, the quantity of plasma cells was very small and the calculations of the plasma cell index unreliable (see the large standard deviation of the plasma cell index in Table 2) The total amount of pyroninophilic cells showed an increase 6 and 9 hours after thyroxin treatment, but this trend did not persist 24 and 72 hours after The ratio between the lymphocytes and the reticular cells was raised 11 per cent 3 hours after, then fell The mitotic indices of the secondary follicles remained unchanged (Table 5) At statistical analysis none of the changes in the cortical layers were found to be significant

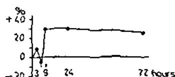


Fig 2

Percentage changes of the relative contents of pyroninophilic cells in the medullary cords of cervical lymph nodes in guinea pigs after treatment with thyroxin

TABLE 5

Mitotic Indices of the Secondary Follicles of the Cervical Lymph Nodes in Thyroxin Treated and Control Animals Mean ± Standard Deviation

	Controls	3 hours	6 hours	9 hours
Mitotic indices	2.3 ± 0.4	2.1 ± 0.5	2.2 ± 0.5	2.2 ± 0.6
Number of animals	7	5	8	8

The *spleen* was examined 24 and 72 hours after the injection of thyroxin In the *red pulp* there was a mean increase of the relative contents of lymphocytes This was reflected by an increase in the ratio of lymphocytes to reticular cells of 23 per cent and 36 per cent, respectively, 24 and 72 hours after The contents of pyroninophilic cells and the plasma cell index were found to be higher after 24 hours, but the changes did not persist after 72 hours None of the changes in the red pulp were significant ($0.05 < p < 0.2$)

The *white pulp* did not differ in relative cell composition from that of normal animals

The thymus In the *medulla* the relative amount of pyroninophilic cells had diminished in all of the groups examined at different intervals after the administration of thyroxin (Fig 3) The decrease was maximal after 6 hours, whereas the percentage of lymphocytes was maximally raised From 9 to 72 hours after injection the relative contents of lymphocytes fell from ±0 per cent to —4 per cent, as compared with

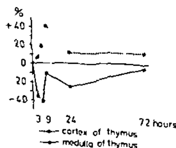


Fig 3

Percentage changes of the relative contents of pyroninophilic cells in the thymic medulla and cortex in guinea pigs treated with thyroxin

the control animals (Table 6) 24 and 72 hours after, the number of the reticular cells went up 18 per cent and 12 per cent, respectively (Table 6)

TABLE 6

Percentage Changes of the Cell Composition of the Thymic Medulla in Guinea Pigs Treated with Thyroxin

Hours after administration of thyroxin	Reticular cells %	Pyroninophilic cells %	Lymphocytes %
3	+4	−36	+2
6	−7	−42*	+5
9	+3	−11	±0
24	+18*	−26	−4
72	+12*	−5	−4

* indicates $p < 0.05$ when individual groups are statistically compared with respective controls

TABLE 7

Percentage Changes of the Cell Composition of the Thymic Cortex in Guinea Pigs Treated with Thyroxin

Hours after administration of thyroxin	Reticular cells %	Pyroninophilic cells %	Lymphocytes %
3	+44	+6	−5
6	+1	+18	−2
9	+12	+40*	−4
24	+6	+11	−1
72	−8	+12	−1

* indicates $p < 0.05$, when individual groups are statistically compared with respective controls

In the *cortex of the thymus* the change in the cell population was contrary to that of the medulla Thus, the amount of pyroninophilic cells was relatively larger in all of the groups examined (Table 7 and Fig 3), and the relative amount of lymphocytes had decreased throughout

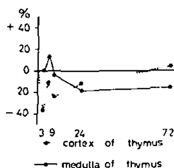


Fig 4

Percentage changes of the ratio between lymphocytes and reticular cells in the thymic medulla and cortex in guinea pigs treated with thyroxin

(Table 7) The percentage of reticular cells was above that of the control animals except in the last group examined after 72 hours (Table 7) The changes in the ratio between lymphocytes and reticular cells in the thymic medulla and cortex, respectively, are illustrated in Fig 4

DISCUSSION

The cervical lymph nodes in young guinea pigs are observed to contain a higher percentage of plasma cells as compared to the extremity nodes The cause is unknown, but broadly speaking two different sup positions have been laid down On the one hand, it may be assumed that, as the lymph nodes rich in plasma cells are situated in the neighbourhood of the thymus, they might receive lymph from the thymus including a great many thymic cells with, possibly, a potency to develop into immunologically competent cells (as indicated by *Kelsall* 1958, quoted by *Fichtelius* 1960) On the other hand, the high percentage of plasma cells in some of the lymph nodes might, conceivably, be due to the abundance of exogenous antigenic substances However, a combination of the two theories is not unlikely A plentiful supply of immunologically competent cells combined with exogenous stimuli may be the cause of the lymph node tissue rich in plasma cells

After the administration of thyroxin to guinea pigs a rapid increase is to be noted of the contents of plasma cells in the *lymph nodes* This effect of thyroxin is apparent in the medulla of the cervical lymph nodes as soon as 3 hours after the hormone injection The maturation effect of thyroxin is not selectively directed to the lymphatic tissue It is well-known that thyroxin hastens the development and regenera tion of several mammalian tissues (for ref see *Nowinski* 1960)

After this, signs of proliferation are seen in the lymph nodes Thus, the total contents of pyroninophilic cells are to some extent enlarged 9 hours after hormonal treatment, and considerably so 1–3 days after The actual growth of the lymph nodes is evident from the investigations by *Gyllensten* in 1953, who examined the weight of the lymph nodes, as well as from *Lundin's* (1958) analysis of the turnover of DNA-P^{32} After the iterated administration of thyroxin the contents of pyroninophilic cells become more and more significant (*Ernström & Gyllensten* 1959) The rapid maturation of the cells referred to above gives rise to a relative shortage of immature cells The increase of these cells later on may constitute the indirect effect of thyroxin, due to some local home [illegible] establishing a normal proportion be- [illegible] lls Evidence of such control mecha- [illegible] scussion by *Osgood* (1959)

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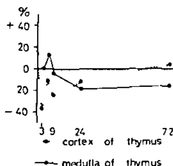


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In the *thymic medulla* the percentage of pyroninophilic cells was reduced throughout the experiments At a maximal decrease 3–6 hours after the administration of thyroxin there was a transitory increase of the relative amount of lymphocytes, followed by a fall in the number

of lymphocytes 24 and 72 hours after it A similar transitory rise in the contents of lymphocytes in the medulla during regeneration in the mouse thymus after hydrocortisone-induced involution has recently been reported (Ito and Hoshino 1962) The slight addition to the quantity of lymphocytes, which is accompanied by a loss in that of pyroninophilic cells, observed in the present investigation material, might be attributed to either an accelerated maturation of pyroninophilic cells to lymphocytes, or to a change in the migration of lymphocytes to and from the thymic medulla The hypothesis of thymic lymphocytes migrating from the cortex to the medulla, before entering the circulation was presented by *Sainte-Marie & Leblond* (1958 b) Such an intrathymic migration of cells may have some bearing on the present observations Still, the results obtained do not permit the drawing of any conclusions as to the justification of such an hypothesis 24 and 72 hours after the administration of thyroxin the relative contents of pyroninophilic cells and lymphocytes were both diminished This change of the cell population may either be due to a proliferation of the reticular cells, or to an altered migration of the lymphocytes to and from the thymic medulla

In the *thymic cortex* the number of pyroninophilic cells was observed to have risen The cortical lymphocytes were, on the other hand, reduced in relative quantities throughout the experimental groups This relative fall in the number of lymphocytes may be attributable either to an increase of other cells, or to an absolute decrease, according to the following alternatives

1) *stimulated proliferation of reticular and/or pyroninophilic cells,*
2) increased transition of lymphocytes to pyroninophilic cells,
3) reduced production of lymphocytes,
4) reduced migration of lymphocytes to the cortex, and
5) increased migration of lymphocytes from the cortex

Firstly, the assumption of a stimulated proliferation of the reticular and/or pyroninophilic cells might conceivably be acceptable, in view of the fact that the cortex of the thyroxin-treated animals microscopically is at least as thick as that of the untreated animals (own observations) It is evident that the change in the contents of pyroninophilic cells undoubtedly represents an absolute increase

In the second case, an opposite opinion has been suggested by most researchers studying the growth and regeneration of the thymus, *viz* that the large pyroninophilic cells should be precursors of the medium-sized and small lymphocytes Thus, the incidence of large lymphocytes with high mitotic indices is great in young animals In old animals, on the other hand, the size of the lymphocytes diminishes (see review by *Kindred* 1955) During regeneration after cortisone-involution the pyroninophilic cells reappear before the medium-sized and small lymphocytes (*Ito & Hoshino* 1962) In 1958 *Sainte-Marie & Leblond* constructed

a tentative pattern to illustrate the formation of lymphocytes from reticular and pyroninophilic cells in the thymic cortex, which was discussed further in 1960. From the above, it emerges that the transition of lymphocytes to pyroninophilic cell types seems to be an unlikely explanation of the cytological changes occurring in the thymic cortex. However, contrasting evidence has been offered of a transformation of small lymphocytes into large pyroninophilic cells (*Gowan et al* 1961, *Porter & Cooper* 1962 a and b). The present author cannot deny the possibility of such an origin of the pyroninophilic cells in the thymic cortex in which case the renewal of lymphocytes would pass through three morphological stages, 1) transformation of the small lymphocytes into large pyroninophilic cells, 2) mitotic division of the pyroninophilic cells, and 3) redifferentiation to small lymphocytes. The ratio of lymphocytes to pyroninophilic cells was decreased even during prolonged treatment with thyroxin (*Ernstrom & Gyllensten* 1959). Therefore, if this renewal theory should be applicable to the present material, an increased migration of cells from the thymic cortex must also be taken for granted.

As regards the third alternative of a reduced production of lymphocytes without any proliferation, this should result in a thin thymic cortex and loss of weight. However, the microscopical examination did not disclose any thinning of the cortical layers which, on the contrary, often looked thicker and richer in cells than did those of the control animals. This was confirmed in earlier studies (*Gyllensten* 1953, *Ernstrom & Gyllensten* 1959) in which thyroxin was found to produce a slight gain in the weight of the thymus by means of doses identical to those used in the present experiments.

The fourth attempt to explain the changes observed in the cell population seems implausible. No reports are available concerning the return of lymphocytes in large numbers to the thymus. If such a recirculation should actually take place to any noteworthy extent, a reduced return would have the same appearance as a lowered production. Thus, a significant decrease in the migration of lymphocytes to the cortical layers seems an improbable theory.

Lastly, concerning the possibility of increased migration of lymphocytes from the cortex, either directly into the circulation or via the thymic medulla, this *might* explain the decreased cortical contents of lymphocytes. As there are no signs of a reduced thickness of the cortical layers nor of a lesser density of cells such a m... [illegible]

... thymic cortex, probably combined with an increased migration of lymphocytes, directly into the circulation, or via the medulla. The theory of an accentuated delivery of cells from the thymic cortex derives support from the finding that prolonged treatment with thyroxin results in a constantly diminished ratio of

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lymphocytes to reticular cells in the thymic cortex, but fails to give rise to signs of lymphocyte destruction (*Ernstrom & Gyllensten* 1959) Whether or not the stimulated activity of the thymus is of any significance for the increase in pyroninophilic cells in the red splenic pulp, as observed during prolonged thyroxin treatment (*Ernstrom & Gyllensten* 1959), is unknown, but it is suggested by the results arrived at by *Fichtelius* (1953, 1958 and 1961), indicating the spleen as a receptor of thymic cells Many reports have moreover recently been made in favour of this, suggesting the thymus to be a source of the immunologically competent cells (*Fichtelius et al* 1961, *Archer et al* 1961, *Miller* 1961, *Martinez et al* 1962, *Dalmasso et al* 1962, *Arnason et al* 1962)

SUMMARY

1 The cell composition of different lymph nodes from young normal guinea pigs is not uniform Especially the contents of pyroninophilic cells and plasma cells are variable, being greater in the cervical lymph nodes than in those of the extremities

2 Thyroxin (50 micrograms/kg body weight subcutaneously) gives rise to pronounced changes in the quantitative cytology of the lymph node tissue in the guinea pig The earliest effect, observed 3 hours after the administration of the hormone, was an increase of the plasma cells as compared to other pyroninophilic cells Then the total contents of pyroninophilic cells were enlarged, probably indicative of a proliferation Such proliferative signs were faintly discernible in the medullary cords of the cervical lymph nodes 9 hours after the thyroxin injection, being much more marked after 24–72 hours

3 Thyroxin gives rise to cytological changes in the thymus In the medulla a fall is seen in the relative quantity of pyroninophilic cells After a transitory rise, the relative amount of lymphocytes diminishes These results may possibly be explained as due to a hastened differentiation of lymphocytes from pyroninophilic cells, followed by a stimulated proliferation of reticular cells and an increased delivery of cells from the medulla

4 In the thymic cortex the amount of pyroninophilic cells was greater and that of lymphocytes smaller after thyroxin treatment These changes may be caused by an intensified proliferation of cells, combined with a stimulated migration of lymphocytes from the thymic cortex, either directly into the circulation, or via the medulla A possible fate of the lymphocytes produced in the stimulated thymus gland is discussed

5 In the red pulp of the spleen a relative increase of lymphocytes was noted 24 and 72 hours after treatment with thyroxin but this change was not statistically significant

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LONG LASTING AMYLOID STAINING TECHNIQUES FOR MUSEUM SPECIMENS

By

PABLO JIMÉNEZ BRUNDELET

Received 27 II 63

As is well-known, when an iodine solution is applied to amyloid organs for a few minutes, the amyloid substance becomes stained a deep brown. If dilute sulphuric acid is subsequently applied to these iodine-stained organs, the amyloid substance turns dark blue or dark green, almost black. These staining reactions which were originally described by *Virchow*, are commonly used in the autopsy room to investigate the possible presence of amyloid in suspected organs (spleen, kidneys, etc.). Unfortunately, when organs so stained are placed in the normally used fixative, the staining gradually fades away, and after a time varying from a few hours up to about one day, the brown and the blue-green-black colours have completely disappeared, the amyloid substance showing again the same appearance as before staining, *e.g.*, translucent whitish-grey nodules in the case of a "sago" spleen. That may be due to the passing back of a too small amount of staining substances from the amyloid substance to the fixative, with a resulting too excessive dilution to be able to produce any staining, and/or to a possible chemical alteration as the organs are stained unfixed in the autopsy room. At the best of our knowledge, there is only one technique published (1) about the staining of amyloid organs for museum specimens. This technique, although good, gives however (when used as it is described) only the brown staining of the amyloid substance. The object of the present paper is to describe two techniques giving each one the two different classical colours and producing a long-lasting staining of the amyloid substance, allowing therefore the mounting of the specimens for the museum.

General Principles

After prolonged (at least one month) fixation of the sliced organs in neutral buffered 10 per cent formol-saline, the amyloid substance is stained with a iodine solution only (brown colour) or afterwards with a sulphuric acid solution (dark blue green colour). The mounting fluid used is, in one method, a special hypertonic solution in order to prevent

to a certain extent the re-solution of the staining substances, and, in the other method, it is paraffin oil (as in the already published technique)

As both methods differ not only in mounting fluids, but also in length of time needed, we shall refer to them as to the "slow aqueous" technique (about 18 hours), and to the "quick oily" technique (about 6 hours)

THE SLOW AQUEOUS TECHNIQUE

Reagents it is advisable to use freshly made up reagents

Iodine solution

Iodine	10 grams
Potassium iodide	250 grams
Distilled water	1000 ml

This solution will stain the amyloid substance brown (If freshly made up Lugol s solution is available the above reagent may be rapidly prepared by adding 230 grams of potassium iodide per litre of Lugol s solution)

Mounting fluid for the brown stained amyloid organs

c	25 grams
	250 ml
	6 1 grams
	3 9 grams
	750 ml

Mounting fluid to obtain the dark blue green stained amyloid organs

Sulphuric acid (concentrated)	50 ml
Sodium chloride	25 grams
Formaldehyde (40 per cent)	250 ml
Distilled water	700 ml

(Here the sulphuric acid is incorporated to the mounting fluid)

Technique

(1) Dip the fixed sliced organs into the iodine solution for about 18 hours (The ratio of volume of staining fluid to volume of organs should be at least 5 to 1)

(2) Mount using the mounting fluid corresponding to the final staining desired (The ratio of volume of mounting fluid to volume of organ in the mounting jar is not critical it may be 2 to 1 or even 1 to 1)

Results

Shortly after mounting, the fluid in the jar will become iodine stained to some extent This will largely fade in the next few weeks or months, but if excessive, the mounting fluid should be changed once or twice The staining of the amyloid substance in the specimens will not fade appreciably for many months If and when it eventually does so, it is merely necessary to unseal a small hole in the lid of the jar, replace the mounting fluid by the iodine solution for 18 hours (or even less time), and to refill with the corresponding mounting fluid It is, of course desirable to have the demonstration surface of the specimens close to the anterior surface of the jar, but care should be taken to avoid its coming into contact with it, since compression appears to produce some fading of the staining Two factors which appears important to

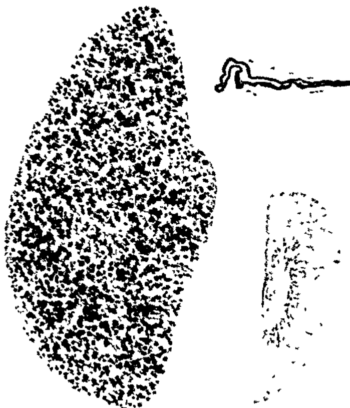


Fig 1

Black and white photographs of mounted *specimens stained with the* "slow aqueous" technique and showing amyloid infiltration of spleen adrenal, and kidney in a case of secondary amyloidosis

ensure a long-lasting staining are' perfectly fixed organs and air-tight jars

THE "QUICK OILY" TECHNIQUE

Reagents

Iodine solution the same as for the aqueous technique
Sulphuric acid solution a 5 per cent aqueous solution is used
Mounting fluid Paraffin oil

Technique

To obtain the brown staining of the amyloid substance

(1) Dip the fixed sliced organs into the iodine solution for about 5 to 6 hours
(2) Dry the surfaces of the organs with blotting paper in order to remove the excess of free aqueous solution
(3) Mount the organs in paraffin oil

To obtain the dark blue green staining of the amyloid substance

(1) Dip (as previously) the fixed sliced organs into the iodine solution for about 5 to 6 hours

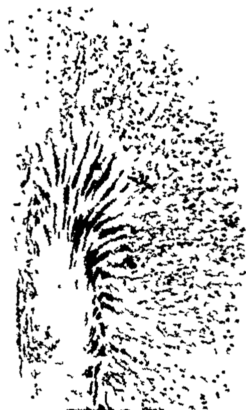


Fig 2

Cl se up view from the previ us specimen t show the characteristic distr bution of the amylo d infiltrati n in the ki lney

(2) Dry (as previously) the surfaces of the organs with blotting paper in order to remove the excess of free aqueous s lution
(3) D p the l rown sta ned rgans into the sulphuric acid solution for 30 mi nutes
(4) Dry the surfaces of the organs with blott ng paper in order to remove the ex ess of free aqueous s lut n
(5) Mount the rgans in paraff n il

Results

Shortly after mounting (or the following day) and due to the action of the sulphuric acid the paraffin oil will become pinkish To decrease partially the intensity of the pinkish staining a perfect drying of the surfaces of the organs with blotting paper is essential in order to remove the excess of free acid solution As an additional precaution and this is also good to deal with the formation of iodine stained drops of water in oil due to imperfect drying of the specimens the organs may be first

placed vertically into a jar containing paraffin oil and mounted a few hours later in the definitive mounting jars using fresh paraffin oil for it

SUMMARY

Two techniques producing a long lasting staining of amyloid organs for museum preservation are described in detail

I wish to thank particularly Mr Tom Lawley Technician from the Department of Pathology University of Manchester (Great Britain) who mounted the specimens shown in the photographies

REFERENCE

(1) *in Culling C F A* Handbook of histopathological technique (including museum technique) page 375 Butterworth & Co Ltd (Publishers) London (1957)

FROM THE INSTITUTE OF CLINICAL BACTERIOLOGY UMEÅ AND THE BLOOD BANK OF THE UNIVERSITY HOSPITAL LUND SWEDEN

EFFECT OF ENDOTOXIN ON EMBRYONAL CHICK FIBROBLASTS CULTURED IN MONOLAYER

By

S. Bergman and S. B. Nilsson

Received 7 iii 63

Smith & Thomas (1956) found endotoxin to have a lethal effect on chick embryos. It was therefore considered legitimate to study the effect of endotoxin on chick fibroblasts cultured on object glasses according to the method of Bergman (1959 63).

MATERIAL AND METHODS

Chick embryos of our white leghorn breed were used. The viscera were carefully

RESULTS

Cells that had been exposed to endotoxin for 4 hours *in the rings* and then cultured for 24 hours yielded carpets of cells easy to count. The chick embryo which was 7 days old gave good growth of the primary cultures. Older embryos also gave *good growth* but they were not so susceptible *to endotoxin*. *Since the investigation* was concerned with subcultures of *chick fibroblasts* attention was first directed to the effect if any of the age *of the primary culture* on the results obtained with the secondary *cultures*. Endotoxin was found to influence the number of mitoses. Four and five day old cultures proved most susceptible; therefore 5-day *old cultures* were used since they were easiest to count and gave the most constant results.

The costs of this investigation were defrayed by grants from the Swedish Medical Research Council and the Swedish Cancer Society.

placed vertically into a jar containing paraffin oil and mounted a few hours later in the definitive mounting jars using fresh paraffin oil for it

SUMMARY

Two techniques producing a long-lasting staining of amyloid organs for museum preservation are described in detail

I wish to thank particularly Mr Tom Lawley, Technician from the Department of Pathology, University of Manchester (Great Britain), who mounted the specimens shown in the photographies

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(1) *in Culling C F A* Handbook of histopathological technique (including museum technique) ', page 375 Butterworth & Co Ltd (Publishers) London (1957)

(*Janoff & Zweifach* 1960) and it is therefore possible that the metal components of Parker 199 might have inactivated the endotoxin.

In titration studies the number of mitoses was found to vary with the amount of endotoxin used (Fig 2). Even such a small amount of endotoxin as 2 γ was found to have an inhibitory effect on mitoses.

The differences between the number of mitoses in the controls in Figs 1 and 2 must be ascribed to differences in the primary cultures which are not uncommon. This may depend on the high sensitivity of chick fibroblasts to trypsin, i.e. that small variation in time and concentration may influence the results.

DISCUSSION

It is not known whether the effect of the endotoxin should be regarded as primary or secondary. Newborn rabbits are refractory to endotoxin (*Gratia & Linz* 1932); not until the animals are several days or weeks old can a Schwartzman phenomenon be elicited. The lethal dose (mg endotoxin/kg bodyweight) for newborn rabbits is larger than for animals weighing 2-3 kg (*Smith & Thomas* 1954, *Witebsky* 1936).

It has been suggested that endotoxin might be an antigen to which the animals are normally hypersensitive (*Stetson* 1959); if so the effect of the injection of endotoxin would be secondary. However, the toxic effect of the endotoxin on 8-12 day old chick embryos argues for the effect being primary rather than secondary. Our findings show that the endotoxin has a direct effect on the fibroblast cells from chick embryos cultured in monolayer. Endotoxin has no effect on the attachment of the cells but, judging from the mitoses, it considerably inhibits growth frequency even in such a low dose as 2 γ when suspended in Hank's but not in Parker 199 or 2 per cent calf serum. This may depend on metals that inactivate the effect of endotoxin.

SUMMARY

[illegible] mitotic frequency. The investigation also included titration tests.

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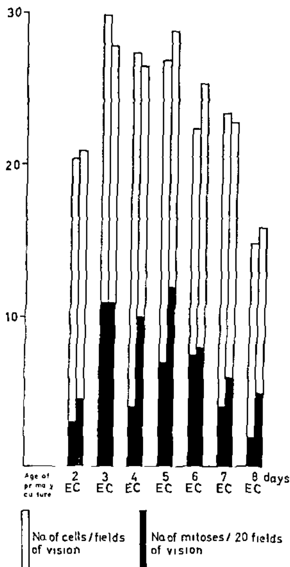


Fig 1
Effect of age of primary culture on results
E = 20 γ Endotoxin in Hank's
C = Control with Hank's

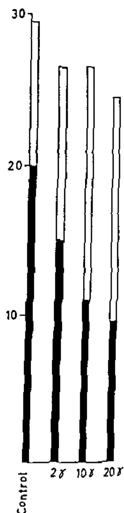


Fig 2
Effect of different doses of endotoxin on results 5 days culture

When the cells were exposed to endotoxin for 2 hours their attachment was poor, and when exposed for 6 hours they appeared to be injured We therefore selected a period of 4 hours for the experiments proper When the cells were first allowed 1 hour to attach before addition of the endotoxin, the results were not better than those obtained by the procedure described above

The cells were suspended in Hank's solution This proved an important point because endotoxin had no demonstrable effect when the cells were suspended in Parker 199 alone or with 2 per cent calf serum It is known that certain metals inactivate the effect of endotoxin *in vitro*

*FROM THE UNIVERSITY DEPARTMENT OF PATHOLOGY
CENTRAL HOSPITAL MALMÖ SWEDEN*

ON CANCER AND POLYPS OF COLON AND RECTUM

By

GORAN EKELUND

Received 8 iii 63

In view of the relatively large number of recent publications on the possible relationship between polyps and cancer of the colon a prevalence survey of these tumours in patients examined post mortem at the Department was considered legitimate Malmo General Hospital is the only hospital in Malmo with its 230 000 inhabitants and practically all (about 99 per cent) patients who die in hospital are autopsied Since most deaths in Malmo occur in hospital and not in the patients homes the majority (60 per cent) of all people dying in Malmo are examined at the Department Our material is thus not a collection of cases referred by specialists or of a group with predominantly diseases of the intestines but a good cross sectional sample of a normal population

MATERIAL AND METHODS

The investigation covers a 3 year period (Sept 1 1958 to Aug 31 1961) during which the intestinal tract was always slit up and examined and any pathological findings made were noted in the protocols If gross inspect [illegible] [illegible] protocols for the 3 year period were perused by the author Of tumours of the colon only cancers and adenomatous polyps were included thus n carc no ls l pomas etc

RESULTS

During the 3 year period covered by the investigation 3 398 cases were autopsied at the Department (Table 1) Of these 3 041 were above 1 year and in these cancer of the colon was found in 5 9 per cent and polyps of the colon in 12 5 per cent The polyps were solitary in 6 8 per cent and multiple in 5 7 per cent of the cases

Polyps were found in 25 6 per cent of the patients with cancer and in 11 0 per cent of those without The coexisting polyps were solitary in 10 per cent and multiple in the remaining 15 6 per cent

The segments most commonly involved by cancer (Table 2) were the

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Witebsky F & Neter E Significance of Age of Rabbits for Elicitation of Shwartzman Phenomenon J Immunol *30* 471 1936

FROM THE UNIVERSITY DEPARTMENT OF PATHOLOGY
GENERAL HOSPITAL MALMÖ SWEDEN

ON CANCER AND POLYPS OF COLON AND RECTUM

By

GÖRAN EKELUND

Received 8 i 63

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TABLE 1

Incidence of Polyps and Cancer of the Colon of the Large Intestine Found at our 3 398 Consecutive Autopsies Performed during a 3 Year Period (1.9.58–31.8.61) at the University Department of Pathology, Malmo General Hospital

Cases	First year		Second year		Third year		Total	
	No	%	No	%	No	%	No	%
Total	1065		1127		1206		3398	
Above 1 year	955		1014		1072		3041	
Without polyps	860		878		923		2661	
With polyps	95	10.0	136	13.4	149	13.9	380	12.5
With solitary polyp	50	5.2	70	6.9	87	8.1	207	6.8
With multiple polyps	45	4.7	66	6.5	62	5.8	173	5.7
With cancer	47	4.9	55	5.4	78	7.3	180	5.9
With cancer but without polyps	37		39		57		133	5.0
With cancer and coexisting polyps	10		16		21		47	
Frequency of polyps (cases) in pat with cancer		19.1		29.0		27.0		25.6
Frequency of cancer (cases) in pat with polyps		9.5		11.8		14.1		12.4
Solitary polyp in pat with cancer	4	8.5	3	5.5	11	14.1	18	10.0
Multiple polyps (cases) in pat with cancer	5	10.6	13	23.6	10	12.8	28	15.6
Solitary or multiple polyps (cases) without coexisting cancer	85	8.9	120	11.8	128	11.9	333	11.0

Unless otherwise stated all percentages refer to no of cases above 1 year

TABLE 2

Distribution of Cancer among Various Segments of Large Intestine

Segment	First year	Second year	Third year	Total	%
Rectum	11	14	18 (+2)	43 (+2)	23.9
Sigmoid	17 (+1)	27	27 (+4)	71 (+5)	39.4
Descending colon	0	0	5	5	2.8
Transverse colon incl flexures	6	5 (+1)	[illegible] (+1)	20 (+2)	11.1
Ascending colon incl caecum	13	8	17	38	21.0
Site not noted	0	1	2	3	1.7
Total	47 (+1)	55 (+1)	78 (+7)	180 (+9)	

Figures in brackets indicate cases with coexisting cancer in other segment

TABLE 3

Distribution of Solitary Polyps among Various Segments of Large Intestine

Segment	First year	Second year	Third year	Total	
				No	%
Rectum	5	19	19	43	20 8
Sigmoideum	17	24	34	73	35 3
Descending colon	3	7	6	16	7 7
Transv incl flexures	9	6	17	32	15 5
Ascend incl coecum	13	13	13	39	18 8
Site not noted	3	1	0	4	1 9
Total	50	70	87	207	

TABLE 4

Distribution of Multiple Polyps among Various Segments of Large Intestine

Segment	First year	Second year	Third year	Total	
				No	%
Rectum	10	11	11	32	11 0
Sigmoideum	13	30	21	64	22 0
Descending colon	10	18	11	39	13 4
Transv incl flexures	17	17	21	55	18 9
Ascend incl coecum	22	27	24	73	25 1
Site not noted	6	9	13	28	9 6
Total	78	112	101	291	

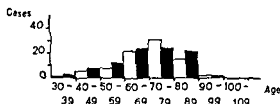


Fig 1

Age and sex distribution of patients with cancer

Filled columns — *females*
Hollow columns — males

sigmoid and rectum (63 3 per cent), and the coecum and ascending colon (24 per cent)

Solitary polyps displayed the same tendency, namely 56 1 per cent in the sigmoid and rectum and 18 8 per cent in the coecum and ascend-

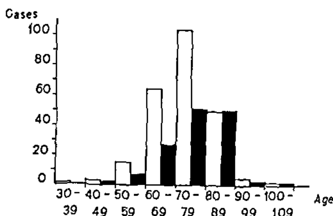


Fig 2

Age and sex distribution of patients with polyps

Filled columns = females
Hollow columns = males

ing colon (Table 3) The distribution of multiple polyps among the various segments was more even (Table 4)

The frequency of cancer (Fig 1) was not found to vary with sex (85 males, 95 females), while polyps (Fig 2) were almost twice as common in males (240 males, 140 females)

The age distribution showed nothing remarkable

DISCUSSION

The figures given above are, if anything, fictitiously low, for though but few cancers can have escaped detection, polyps, especially if small and of the same colour as the contiguous mucosa, are readily missed The increasing frequency of polyps found from one year to the next is probably ascribable to increasing carefulness of the search for them The true frequency is probably even higher than that found for the third year of the study

In the examination of the operative specimens, interest had probably been focused on the cancer with the result that a relatively larger number of polyps might have escaped attention This is clear from the fact that the frequency of polyps found for the operative specimen and/or residual intestine of the patients operated upon for cancer was only 19 1 per cent (17 of 89) against 33 0 per cent (30 of 91) for the cancer patients who had not been operated upon

The incidence of polyps and cancer in published series varies widely, the variation being due in part to the fact that most of the series consist of more or less selected cases The cases have often been selected according to age The examination methods used also vary widely and range from proctosigmoidoscopy and roentgenography to post mortem examination with a magnifying lens (*Atwater* cit by *Rider et al*) (6)

In their compilation of some published series of polyps found at necropsy *Rider et al* (6) found frequencies ranging between 2 6 per cent and 21 4 per cent and in one series 69 per cent (magnifying lens)

In a series of 3 609 patients studied roentgenographically at Malmo General Hospital *Andren & Frieberg* 1959 (1) found polyps to occur in about 10 per cent of all patients examined *Blatt* (2) reported a frequency of 38 8 per cent in an autopsy series of 446 patients above 30 years and Helwig reported an incidence of 9 5 per cent in one autopsy series of 1 460 cases and of 10 4 per cent in another equally large series

The incidence of polyps in our material, 12 5 per cent (12 7 per cent in patients above 30 years), is thus not unusually high but roughly the same as that in Helwig s series As mentioned previously, our figures must be regarded as minimal values but nevertheless fairly representative of a normal population

In half of our cases with polyps the latter were solitary, which is in good agreement with the findings of *Helwig* (4), while *Blatt* (2), for example reported that 58 per cent of his cases had more than one polyp

The distribution of cancer and polyps among different segments of the large intestine also varies from one series to another Thus *Blatt* (2) found 23 per cent of the polyps in his series to be situated in the transverse colon and 27 per cent in the sigmoid rectum while most authors give a frequency of 70 per cent to 80 per cent for the sigmoid rectum (3)

In the present series both cancer and polyps were most common in the distal part of the colon

A noteworthy finding in our series was that the distribution of cancer was almost identical with that of solitary polyps (Tables 2 and 3)

The higher frequency of polyps found in males than in females was in good agreement with the results obtained by previous investigators (5 6) *Blatt* (2) however, found no definite difference with sex

A question which has received much space in the literature is whether any relationship exists between cancer and polyps of the large intestine Most authors believe in the existence of such a relationship and many feel that cancer develops from previously benign polyps This view, however is not shared by *Spratt, Ackermann & Moyer* (9), who assert that the distribution of cancer and that of polyps are not congruent and stress that they were unable to demonstrate any rests of polyps in their cancers and therefore think that cancer most likely develops directly from the mucosa *Ells* (3) however described some cases in which the cancer contained rests of polyps He also found a close resemblance between the distribution of cancer and that of polyps which argues for a relationship *Helwig* (4) studied serial sections of the tumours in his series and considered the histological findings to warrant the conclusion that cancer can develop from adenoma

The observations made in the present material also argue clearly for a causal relationship between cancer and polyps the incidence of cancer in patients without polyps being 5 per cent against 12 4 per cent in those

with coexisting polyps, and cancer being about 50 per cent more common in patients with multiple polyps than in those with a solitary polyp The material would not allow of further analysis in this respect

SUMMARY

In a prevalence survey of cancer and polyps of the large intestine in a series of 3,398 consecutive autopsies representative of a normal population,

polyps were found in 12 5 per cent and cancer in 5 9 per cent of all subjects above 1 year,

polyps as well as cancer were more common in the distal part of the colon,

polyps were more common in males than in females,

the distribution of cancer among the different segments of the intestine coincided with that of solitary polyps, and cancer was more than twice as common in the presence as in the absence of coexisting polyps

These findings appear to warrant the conclusion that polyps of the colon and rectum are related to cancer

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FROM THE DEPARTMENT OF PATHOLOGY POSTGRADUATE MEDICAL SCHOOL OF LONDON

THE AFFINITY OF SOME NAPHTHOLS FOR TISSUES BEFORE AND AFTER EXTRACTION OF LIPIDS

By

JOHAN AHLQVIST[1]

Received 14 iii 63

Tissue lipids seem to have a non enzymatic effect on the quantity of final reaction product formed or deposited in tissue sections in some histo enzymatic methods (*Wolfgram* 1961, *Ahlqvist* 1963) The problem should be studied from many angles The affinity of α-naphthol and some poorly soluble naphthols of the AS series for tissues before and after extraction of lipids from the sections will be dealt with in this paper The results have briefly been mentioned earlier (*Ahlqvist* 1963)

Defendi (1957) in his studies on the affinity of some naphthols for tissues did not mention staining of fat *Hess & Pearse* (1961/62) found that some naphthols in strongly alkaline solutions were absorbed, among others, by lipids and lipo protein structures They did not carry out more extensive lipid extractions and regarded the affinity of these naphthols for epithelial cells and some other structures as due to their substantivity for protein When the present study was completed it was observed that *Wolfgram* (1961) had found that the affinity of some halogen substituted naphthols for myelin sheaths was abolished by preceding extraction with chloroform He regarded this affinity as due to lipid solubility of the naphthols and his findings point in the same direction as the results of the present investigation

MATERIAL

Composite unfixed frozen blocks of rat kidneys and mouse ears were used After killing the animals by exsanguination under light aether anaesthesia a piece of the mouse ear and of the rat ureter with its adjoining fatty tissue were inserted into a cut made in a 5 mm thick slice of the rat kidney Some [illegible] into [illegible] were [illegible] tions

[1] The author was a British Council Scholar
Present address Departm of Pathology University of Helsinki Helsinki Finland

OBSERVATIONS

Affinity of Naphthols for Tissues

α-naphthol—It is comparatively easy to get differential staining of certain structures (see below) in sections kept in saturated or nearly saturated solutions of α-naphthol in tap water or in buffers around pH 7, provided that the time of washing the sections in water before coupling with the diazonium-salt (Fast Blue B, tetrazotized o-dianisidine) is short enough The method was standardized as follows

Introduce 0 1 ml of a 2 per cent solution of α-naphthol in acetone through a submerged pipette into 5 ml of tris buffer, pH 8 8, diluted with 7 5 ml of distilled water After 5–10 seconds pour the solution into a small Coplin jar containing the sections mounted on coverslips and previously wetted in the same buffer Keep the sections in the solution for 2–5 minutes Transfer the jar to a larger vessel of running tap water without first pouring off the naphthol solution Keep in running water for 1–2 minutes, pouring off the water twice Remove the jar, pour off the water and couple with 30 mg of Fast Blue B in 10 ml of the above buffer After one minute the sections are washed in running water This method has been worked out from the method for non-specific esterases used by *Schnitka & Seligman* (1961) The sections may be rinsed in 1 per cent HCl in 30 per cent ethanol

By this method the tubules in the kidney cortex stained strongly and the glomeruli were pale In the outer medulla thick ascending loops stained well, whereas thin loops of the medulla were virtually devoid of pigment Depot fat, cartilage fat and sebaceous glands contained heavy deposits of azo dye and nerve sheaths stained well The basal layer of the epidermis, the hair follicle epithelium and certain granules and streaks in the superficial layers of the epidermis sometimes were pigmented this staining was variable, however Other structures in the ear stained either very faintly or not at all

Naphthols AS-D, AS-BG, and *AS-OL*—No staining was obtained with naphthol AS D (2-hydroxy 3 naphthoic o-toluidide, Brenthol OT, I C I, Ltd) or naphthol AS–BG (2-hydroxy-3-naphthoic-2' 5'-dimethoxyanilide, Brenthol FO, I C I, Ltd) in aqueous buffers around pH 7 This must be attributed to their poor solubility in water the addition of Fast Dark Blue R (Diazoecht dunkelblau R, Rohner S A , Prattein) to the filtrate of a mixture of these naphthols in water gave no reaction, but a strong blue colour developed if a few drops of acetone had been added before filtration In preliminary tests, therefore a few mg of these naphthols were dissolved in 0 25 1 ml of acetone and/or dimethylformamide and these solutions were diluted with tris buffer at pH 7 2 to make 10 ml Sections kept in these solutions for 10 25 minutes, washed in running tap water and exposed to Fast Dark Blue R, Fast Red Violet LB or Fast Red 3 GL gave weak positive reactions in those structures which also stained with α-naphthol and Fast Blue B

The affinity for fat and the poor solubility of these naphthols in water gave rise to attempts to use them in the same manner as the usual Sudans. A saturated solution of naphthol AS-D in 70 per cent ethanol was used. Sections were treated in the manner used for staining of fats with Sudans, and after they had been brought back to water they were exposed to Fast Dark Blue R. In this way results resembling the ones above were obtained. Sometimes also other cellular structures showed positive reactions. A similar staining pattern was obtained when sections were treated with cold saturated solutions of naphthol AS-D in acetone. In this case the sections often were dirty, however, and in addition muscle, for example, stained a dark brownish grey and the results were difficult to interpret.

It was easier to obtain clear results if a few mg of naphthols AS-D and AS-BG were dissolved in dimethylformamide and the solutions were diluted with ethyl cellosolve (Ethylene glycol monoethyl aether), tris buffer at pH 7.2 and finally with distilled water. This technique was worked out from the method for non-specific esterases with naphthol AS-LC acetate described by *Burstone* (1957). It was subsequently found that more intense staining was obtained by adding the water dropwise until it became difficult to keep the solution clear by shaking and that the staining improved if the solution when it became more turbid, was substituted by a freshly made one. Very good results were also obtained with naphthol AS-OL (2-hydroxy-3-naphthoic o-anisidide, Brenthol FR, I.C.I. Ltd.) according to these principles. The method was standardized as follows.

Dissolve 15 mg naphthol AS-D (30 mg of AS-BG or 15 mg of AS-OL) in 2.4 ml dimethylformamide. Add 6 ml of ethyl cellosolve and 6 ml of tris buffer pH 7.2. This solution stays clear. Immediately before use add distilled water (Naphthols AS-D and AS-BG about 1 ml, AS-OL about 3 ml) dropwise while shaking to 5 ml of this stock solution. The solutions which may turn opalescent when water is added should clear upon shaking, a slight opalescence may remain. These solutions are poured upon the sections mounted on coverslips kept upright in small Coplin jars. After about 10 minutes the solutions usually turn more turbid, then a new staining solution is made from the stock solution. The old solutions should be replaced rapidly without letting the sections dry. The procedure was repeated at least twice during 30 minutes. After this the whole Coplin jar is put into a larger vessel with running water without first removing the naphthol solution. For a few seconds a gentle stream of running water is then directed into the jar. The sections generally were washed for 1/2 to 1 minute, this time may be prolonged. After washing coupling is performed with 1 mg of Fast Dark Blue R in 10 ml of acetate buffer pH 5.6, for about 10 minutes. For reasons unknown the staining sometimes seemed to be less intense if stronger solutions of the diazonium salt were used.

Using this method a strong blue reaction was observed in depot fat,





1

2

Figs 1 2

cartilage fat and sebaceous glands of the mouse ear Nerve sheaths stained well A weaker positive reaction occurred in the basal layer of the epidermis and in the hair follicle epithelium Occasionally a weak reaction was obtained in the walls of the smaller vessels In the superficial parts of the mouse epidermis there were areas with a granular or streaky deposit of the azo dye and sometimes dye granules were present in the cytoplasm of connective tissue cells Collagen did not seem to stain and there was no clear positive reaction in cell nuclei In cross-striated muscle azo dye was deposited as small dots and occasionally there seemed to occur staining of certain areas at the periphery of the muscle fibers

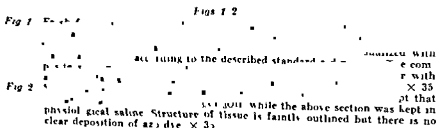
In the cortex of the rat kidney a fairly strong reaction was present in proximal and distal convoluted tubules Glomeruli stained very weakly Sometimes a brushborder-like staining was seen Thick ascending loops in the outer medulla stained fairly well, thin loops in the medulla very poorly A slight deposition of azo dye was present in the epithelium and in the smooth muscle of the ureter The cytoplasm of connective tissue cells in the lamina propria occasionally gave a weak reaction The findings are illustrated in Figures 1 and 3–5

The results obtained with this method were fairly constant The staining of usually weakly stained structures varied slightly This probably depended on variation in the concentration of dissolved naphthol in the solutions Staining with naphthol AS OL might have been somewhat stronger than with naphthols AS-D or AS BG

Effect of Lipid Extraction on Naphthol Staining

Extractions were performed on sections only These had either been dried or post fixed in cold formol calcium Extractions were performed with hot acetone or hot chloroform methanol (2/1, v/v) in Coplin jars, the temperature of the water-bath being 59° C The extracting solutions were changed 3 times irrespective of the duration of extraction Sections to be compared were cut at the same time and always stained in the same solutions If 2 sections only were to be compared they were placed standing face to face in the Coplin jars during the staining procedure

α-naphthol —The staining of cartilage fat, depot fat, sebaceous glands

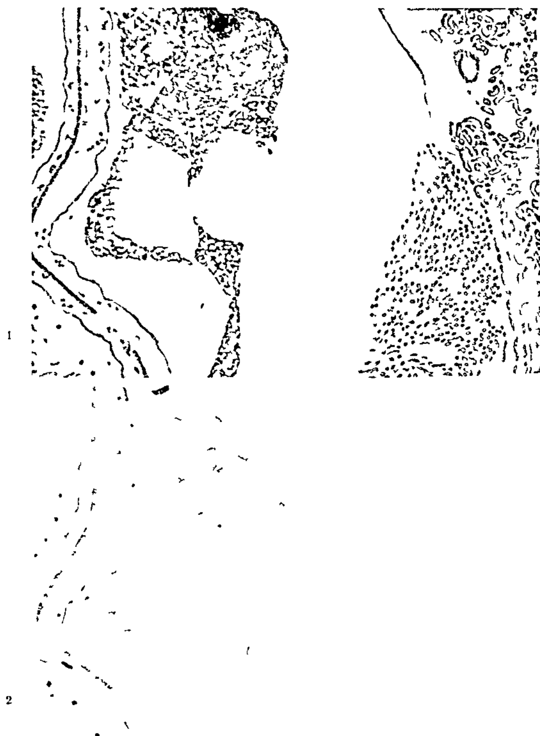


Figs 1 2

Fig 1 ... according to the described standard ... ...dualized with ... e com... r with ... × 35

Fig 2 ... ...ot that ... while the above section was kept in physiological saline Structure of tissue is faintly outlined but there is no clear deposition of azo dye × 35

1



2

Figs 1 2



Fig 5

Detail of section of mouse ear in Fig 1 Azo dye visible as small dots in cross striated muscle There seems to be a concentration of dye at the periphery of some muscle fibers × 970

and nerve sheaths was always abolished by extraction with hot chloroform methanol for half an hour irrespective of whether the sections had been post fixed in cold formol calcium or not A positive reaction sometimes seen in the superficial layers of the epidermis seemed to be abolished by this extraction Staining of the basal layer of the mouse epidermis mouse hair follicles cortical tubules in rat kidney, ascending limbs in the outer medulla and other occasionally positive cellular elements was diminished by the extraction Some affinity always seemed to remain however especially if longer impregnation times had been employed Thus the effects of the extraction on the affinity of α naphthol for tissues are not very clearcut

Naphthols AS D AS BG and AS TR —These poorly soluble naphthols do not seem to be absorbed by the sections after extraction with hot chloroform methanol The structure of the tissue was outlined in a faint grayish brown colour No clear positive reaction was observed Fig 2)

Serial sections were also extracted in different ways One of these tests will be described Four fresh frozen serial sections were dried for 3 hours then one section was dried for a further ½ hour one was treated with cold and one with hot acetone and one section with hot chloroform methanol for the same period of time The sections were



Figs 3 4

Fig 3 Detail of kidney cortex from section in Fig 1 Deposition of azo dye is evident the glomeruli are pale and the nuclei appear to be unstained × 385

Fig 4 Detail of section of mouse ear in Fig 1 Pigment is present in the basal layer of epidermis the sebaceous glands and the nerve below the sebaceous gland to the left Cellular elements in connective tissue and wall of small vessel in the left lower quadrant are stained faintly × 385

Hess & Pearse (1961/62) believe that the final distribution of pigment may be an artefact produced in part by selective substantivity of either coupling agent towards certain histological structures

If we extend Defendi's reasoning we must assume that the amount of azo dye deposited in the tissue in histo-enzymatic reactions will vary according to conditions favouring the attachment of the naphthol to the tissue *Wolfgram* (1961) assumes that a decreased disposition of azo dye in myelin sheaths after extraction of the lipids is due to an escape of liberated naphthol into the incubating medium The magnitude of such an escape from *e g*, fatty tissue from which lipid has been removed (*Ahlqvist* 1963) must be tremendous, however, and the point should be more thoroughly studied If we assume that depot fat does not contain enzymes (cf *Holt & Withers* 1958), that enzymes are not removed by brief exposure of tissues to lipid solvents, and that in, for instance reactions for esterases no tremendous escape of liberated naphthols occurs after the removal of fat from, *e g*, fatty tissue (*Ahlqvist* 1963), corpora lutea (*Hunter & Kneiske* 1957) or sebaceous glands (*Findlay* 1955), what other explanations can we offer for the decreased deposition of final reaction products under such conditions?

The introduction of various sidegroups into the naphthol nucleus influences its solubility, and the AS naphthols belong to the least soluble ones (*Burstone* 1958) Such sidegroups also seem to influence the solubility of the esters of naphthols This is reflected in the use of organic solvents in incubating media with esters of many AS naphthols The present investigation clearly has shown that some AS naphthols, when present in sufficiently highly concentrations (achieved by using organic solvents in the solutions), tend to concentrate in lipid, and it seems reasonable to assume that the corresponding esters, although apparently somewhat more soluble, show a similar tendency The contrary, *i e*, that the AS esters would achieve the same concentration in all parts of the section or that such a differential distribution would differ extensively from that of the corresponding free naphthols seems less probable At least as regards many lipases there may be an intimate relationship between lipid and enzyme, and it seems plausible to assume that the concentration of some naphthol AS acetate at the site of an esterase metabolizing lipids might be higher when lipid is present than when it has been removed It does not seem illogical that such a high concentration could lead, by a pure mass law effect, to the liberation or more free naphthol than in the absence of lipid This could offer an explantion to decreases in "esterase activity" after removal of lipids by, for instance acetone or paraffin embedding It is not known whether this affects other enzymes as well, it does not seem impossible, however, since many enzymes are associated with lipid-containing structures and membranes If this hypothetical mechanism plays a rôle in azo dye methods there does not seem to be any reason why it could not do so in other histoenzymatic reactions as well

then dried for 8 hours and stained with naphthol AS–D and Fast Dark Blue R according to the standardized procedure The result in sections which were not extracted have already been described After cold acetone the reaction in depot and chondrocyte fat had disappeared the reaction in the sebaceous glands was weakened, other reactions were unimpaired After hot acetone a fairly strong reaction was still present in some kidney tubules, in some of the brushborder-like structures, in the basal epidermis, in smooth muscle, and in some nerves No reaction persisted in the sections extracted with hot chloroform/methanol It may be mentioned that treatment with cold acetone often seemed to have a slightly enhancing effect on the staining of epithelial cells, nerves and some other structures

DISCUSSION

Absorption of naphthols AS–D, AS–BG and AS–OL by many structures in the sections was evident Since the staining was abolished by the previous extraction of lipids and since the stainability of the different structures decreased at least in part in the order in which the lipids (neutral fats, myelins, phospholipids, etc) might be expected to decrease, it is suggested that an affinity for lipid is involved As regards α-naphthol, the affinity for depot fat, sebum and myelin is evident, to what extent the staining of other structures is governed by an affinity for lipids is difficult to assess

It should not be forgotten that diazonium salts seem to have some preference for many of the structures studied There does not, however, seem to be a higher concentration of Fast Dark Blue R in lipid than in other structures and, moreover, the affinity of the tissue for this salt seems to be but slightly affected by extraction with chloroform/methanol It is accordingly believed that the staining with naphthols AS–D, AS–OL, AS–BG and Fast Dark Blue R depends on the affinity of the naphthols for lipid, and furthermore that they behave like many Sudans, *i e*, that they dissolve in the lipids This agrees with the findings of *Wolfgram* (1961) From the Sudans the naphthols may differ, however by showing a comparatively greater affinity for lipids other than triglycerides

The discussion is based on the presumption that the sole effect of the extraction was to remove lipids This seems to be a fairly generally accepted view It cannot be denied, however, that it also might alter the density and other properties of the tissue and thus affect the staining in agreement with facts pointed to by *Baker* (1958)

In the experiments with the AS naphthols, staining was seen in most of the structures in which positive reactions for, *e g* esterases and acid phosphatases usually are obtained with azo dye methods *Defendi* (1957) states that it would perhaps not be possible to demonstrate a hypothetical enzyme in structures for which naphthol has no affinity

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SPLENIC MORPHOLOGY IN IDIOPATHIC TROMBOCYTOPENIC PURPURA, LACK OF ACCUMULATION OF PHOSPHOLIPIDS

By

EIVIND MYHRE and CARL DITLEF JACOBSEN

Received 23 III 63

Slight, but relatively constant histological changes of the spleen have been described in patients suffering from idiopathic thrombocytopenic purpura (ITP) (1, 2, 3, 4) The follicles have enlarged germinal centers containing numerous immature lymphocytes Neutrophils and eosinophils in increased number and megacaryocytes are found in sinuses and pulp Small particles regarded as degenerated platelets have also been described (5) Recently, phospholipid accumulation in histiocytes of the splenic pulp has been reported in seven patients with ITP by *Saltzstein* (6) and in nine patients with this disorder by *Landing et al* (7)

In order to study the significance of the occurrence of lipid-containing macrophages of the spleen in ITP, careful histological re-examination of the spleen was carried out in 39 patients who had their spleen removed from 1951 to 1961

The microscopical changes were slight and inconstant Some specimens revealed large germinal centers with immature lymphocytes as well as megacaryocytes, and increased number of leukocytes in pulp and sinuses Only one case displayed lipid containing macrophages in the splenic pulp

CASE REPORT

A six year old girl had suffered from relapsing cystopyelitis since August 1960 During treatment with sulphonamides she developed [illegible] connection [illegible] ocytopenic [illegible] per cmm [illegible] The num [illegible] as u[illegible] and it was no sign of system[illegible] electrophoresis of the [illegible] 7 months [illegible] it was 700 000

If this reasoning has a real background it seems that care should be exercised in drawing conclusions concerning the quantity of enzyme present at a given site from the amount of final reaction product deposited. Differences in the solubility of various substrates might influence among others the speed of reactions and the quantity of reaction products formed.

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hypercorticoid states as in Cushing's syndrome As far as we know no study have been carried out that can throw light on this question

SUMMARY

Accumulation of lipid containing macrophages in the splenic pulp has been described in one case out of 39 patients with thrombocytopenic purpura The rarity of the finding is stressed

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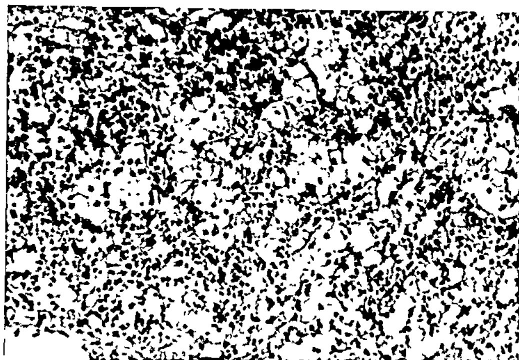


Fig 1
Accumulation of lipid macrophages in the splenic pulp × 320

The spleen weighed 50 grams On histological examination the most prominent feature was a high number of large, foamy and faintly stained lipid macrophages in the pulp (Fig 1) Their lipid nature was confirmed in Sudan stained frozen section

DISCUSSION

Thirty-eight patients with clinical ITP did not have lipid containing macrophages in the removed spleens

The only case with lipid macrophages in the splenic pulp was probably not of the idiopathic (immunological) type, but rather a secondary form, possibly drug-induced Large doses of corticosteroids had been given to this patient for several months

Saltzstein, who observed seven cases with lipid macrophages out of 137 ITP patients, discussed the specificity of this finding He did not observe any case with lipid macrophages in 600 spleens removed for other diseases The cause of the lipid accumulation is not known It is of interest that steroid therapy had been given in the present case and in six of the seven cases reported by *Saltzstein* (6) *Landing et al* (7), however, found the assumption that steroids may produce the splenic changes unlikely as one of their patients had not been treated with steroids Furthermore, experiments in rats given methylprednisolone, 1 mg intramuscularly per day for one or two weeks did not suggest that splenic changes should be expected as sequel of steroid treatment It would have been of interest to study the splenic morphology in other

hypercorticoid states as in Cushing's syndrome As far as we know, no study have been carried out that can throw light on this question

SUMMARY

Accumulation of lipid containing macrophages in the splenic pulp has been described in one case out of 39 patients with thrombocytopenic purpura The rarity of the finding is stressed

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7 *Landing B H*
& Sanders [illegible]
New Engl [illegible] 1961

FROM INSTITUT FOR GENERELL OG EKSPERIMENTELL PATOLOGI (HEAD: PROFESSOR LEIV KREYBERG, M.D.), UNIVERSITETET I OSLO RIKSHOSPITALET

MALIGNANT MELANOMAS IN CHILDREN

By

EIVIND MYHRE

Received 28.iii.63

Malignant melanomas may occur at any age but are extremely rare in childhood. It is well known that the histological picture of moles in children may closely simulate or be indistinguishable from that of malignant melanomas in adults. The benign juvenile melanoma has been claimed to be distinguishable histologically from adult malignant melanoma by the presence of giant cells in the former (2). In spite of the histological resemblance of juvenile melanomas to malignant melanomas a malignant course is very seldom seen in the juvenile type (1, 3).

During the 10 year period from 1953 to 1962 1014 malignant melanomas were reported to the Cancer Registry of Norway, four of them occurring in the age group 9–14 years. Three of the latter were diagnosed in our laboratory and are shortly reviewed below.

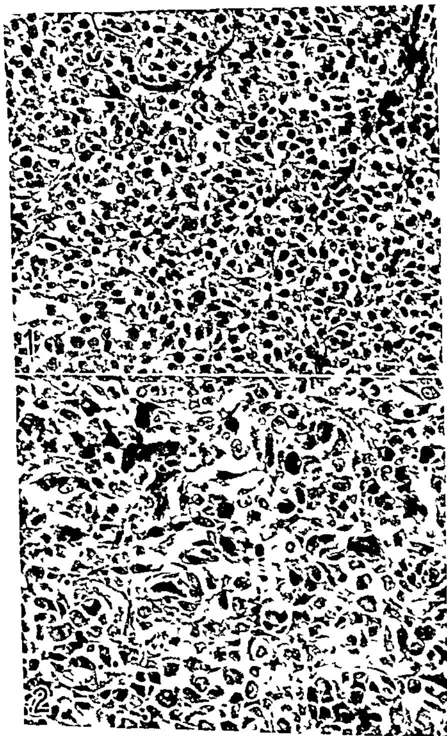
CASE REPORTS

Case No. 1. Boy.

A mole on the back had been present since birth. From about 13 years of age a slight growth of the mole was observed. The mole was removed by excision when the boy was nearly 14 years old. The tumour had the size of a pea. The histological picture (Fig. 1) was that of a heavily pigmented malignant melanoma with great pleomorphism of the cells and many mitoses. An enlarged lymph node of his left axilla was removed four and a half month later, the histology revealing metastasis from the melanoma (Fig. 2) with heavy pigmentation. The patient was given 5 000 r of X-ray treatment. Axillary lymph node toilette was performed two months after removal of the lymph node metastasis, but no more metastases were found. However, a subcutaneous nodule was excised three months later and histologically verified as a metastasis. He died of metastases three years after removal of the primary tumour.

Figs. 1 and 2.

Fig. 1. Case No. 1. Primary tumour with great cellularity and many mitoses. × 320.
Fig. 2. Case No. 1. Axillary lymph node metastasis showing great pleomorphism of the tumour cells. × 320.



1
2

Case No 2 Girl

She had had a slightly elevated dark mole on her back since birth At the age of 9 years the mole started to grow slightly Itching was present and the mole was painted with silver nitrate Excision was performed when she was 10 years old Histological examination showed a malignant melanoma (Fig 3) Pleomorphism of the nuclei and a high number of mitoses were the most prominent features Eleven months later the parents noted a tumour in the scar, histology revealing recidiva tion At the same time metastases were found in axillary lymph nodes of both sides (Fig 4) The metastases displayed very little pigmentation while the primary tumour was rather heavily pigmented Several metastases developed subsequently Death occurred two and a half years after removal of the primary tumour

Case No 3 Girl

A mole on her back had been present since birth A slight growth was observed before the tumour was excised two months before her 10th birthday The pea sized tumour was then elevated with rubor of the surrounding skin Histologically a highly cellular, slightly pigmented malignant melanoma was found (Fig 5) Pleo morphism and mitoses were prominent She is living well ¾ year after excision of the tumour

COMMENT

It has been a tendency to classify juvenile melanomas as a separate entity owing to the benign course despite the histological similarity to malignant melanomas in adults (1, 2) Some authors (1, 2) have claimed that cytological details make the diagnosis of the benign juvenile melanomas possible The three reported cases showed none of these features All three cases displayed histological pictures of frankly malignant melanomas as they appear in adults

The importance of being aware of the rare occurrence of malignant melanomas in children is emphasized The histological criteria of malignancy in the three reported cases were the same as those used in the evaluation of malignancy in adults According to the Cancer Registry of Norway malignant juvenile melanomas occurred in 0 4 per cent of all melanomas in a 10 year period

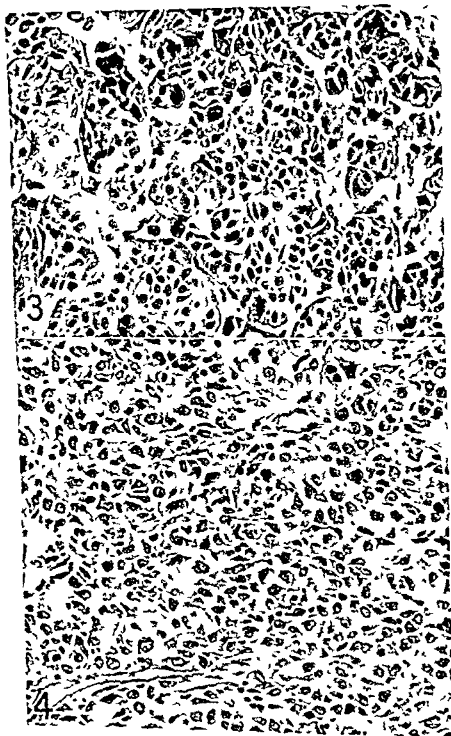
SUMMARY

Three malignant melanomas in children, a boy 13 and two girls, 10 and 9 years of age respectively, are reported Two of them died from metastases, the third is living well ¾ year after excision of the tumour All tumours had been present since birth, and all tumours occurred on the back of the children The importance of being vigilant to this tumour in children is emphasized

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Figs 3 and 4

Fig 3 Case No 2 Primary tumour revealing pleomorphism and mitoses × 320
Fig 4 Case No 2 Axillary lymph node metastasis The pleomorphism is not as pronounced as in Case No 1 × 320


3
4

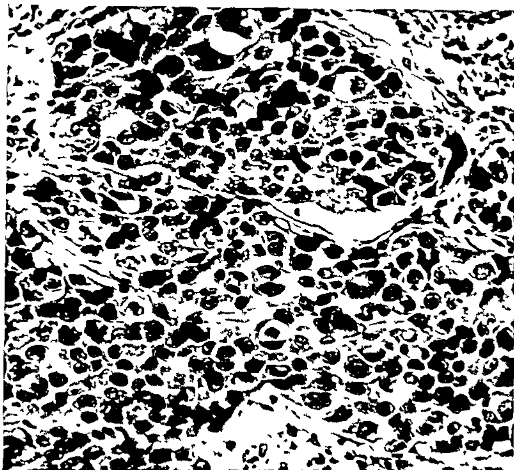


Fig 5

Case No 3 Primary tumour showing pleomorphism and mitoses × 320

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CHRONIC INFLAMMATION OF THE URINARY TRACT RESEMBLING NEOPLASTIC GROWTH

By

EIVIND MYHRE

Received 1 iv 63

In 1961 we had the opportunity in our laboratory to observe a peculiar inflammatory lesion occurring in three different patients all of them clinically suspect of malignant tumours in various organs of the uro genital tract A short history is given below for each patient

CASE REPORTS

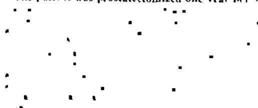
Case No 1 Male 61 Years Old

He had had periodical attacks of dysuria pyuria and fever for two years and micturation disturbance for one month before prostatectomy was performed Clinically there was a strong suspicion of malignancy The specimen obtained from the operation consisted of nine pieces from pea to walnut size The microscopical picture was rather uniform in most areas (Fig 1) dominated by ...

Pathological diagnosis Prostatitis of peculiar type with predominance of lipid macrophages

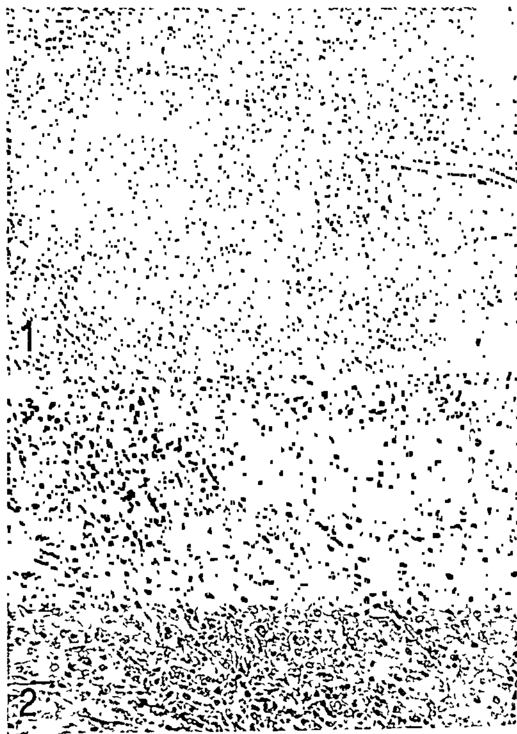
Case No 2 Male 63 Years Old

The patient was prostatectomized one year before the ...



Case No 3 Female 51 Years Old

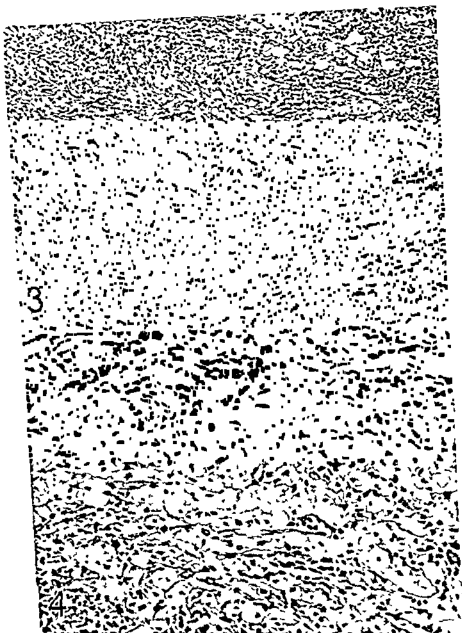
This patient had haematuria for 15 years Several tumours had been removed from the bladder Each time the pathological diagnosis was granulation tissue and chronic inflammation Cancer of the bladder was diagnosed clinically The bladder



Figs 1 and 2

Fig 1 *Case No 1* Rather uniform appearance of the prostatic gland × 125

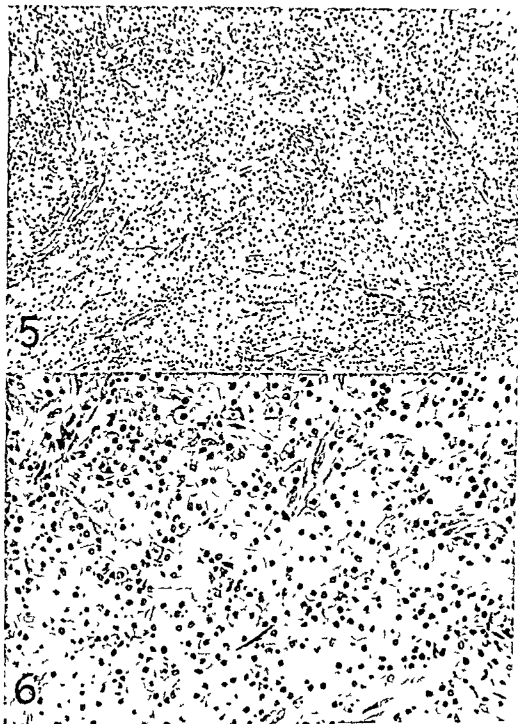
Fig 2 Same case as Fig 1 The tissue is dominated by lipid macrophages Polymorphonuclears and lymphocytes are also present × 120



Figs 3 and 4

Fig 3 Case No 2 The renal structure is destroyed and replaced by connective tissue with many large pale cells and leucocytes × 125

Fig 4 Detail from Case No 2 showing predominance of lipid macrophages × 320



Figs 5 and 6

Fig 5 Case No 3 *Uniform picture of the bladder tumour* This tissue is also dominated by large pale histiocytic cells × 125

Fig 6 Detail from Case No 3 with predominance of lipid macrophages × 320

uterus both ovaries and infiltrated tissue of the pelvis were removed surgically The specimen from the operation was received en bloque The bladder contained a broad based papillomatous tumour The other organs did not demonstrate pathological changes

Pathological diagnosis of the bladder tumour Chronic inflammation with lipid macrophages (Figs 5 and 6)

COMMENT

In spite of different localization of the reported lesions the three cases demonstrate certain similarities All three patients were strongly suspect of malignant diseases clinically and were therefore operated upon Furthermore all lesions appeared in the uro genital tract *viz* prostate gland kidney and bladder The gross appearance confirmed the suspicion of neoplasia The cut surface of all specimens was homogenous and yellowish Microscopical examination only could demonstrate the inflammatory nature of the lesions which are considered to be a peculiar type of chronic inflammation with predominance of large lipid laden macrophages However, no atypical cells were present The organs involved were distructed by proliferation of the macrophages

Scherrer (2) described in 1951 a specimen very similar to the prostate specimen of Case No 1 Scherrer called the lesion Tumorähnliche Wucherung histiocytarer Phagocyten bei chronischer Prostatit He discussed the problem of inflammation versus neoplasia and regarded the lesion as a particular manifestation of chronic inflammation The large cells were interpreted as phagocytic histiocytes A lesion which is most probably similar to the lesion reported by Scherrer and the present author was described in 1920 by *Kinoshita* as carcinoma xanthomatodes prostatae (1)

It is well known that lipid macrophages occur in some inflammatory lesions They may be so abundant that the involved organ or tissue takes a characteristic appearance as it is often seen in the gall bladder The phenomenon is however rare in the genito urinary tract Why it occurs in some cases is obscure The difference from other chronic inflammations is quantitative only and characterized by predominance of macrophages

This rare lesion seems to be of some importance as a differential diagnostic possibility as judged from the three reported cases Most probably the therapy should be conservative possibly conservative surgery

SUMMARY

Three cases of a peculiar chronic inflammation occurring in the genito-urinary tract are reported The lesion is characterized by a high predominance of lipid macrophages giving the tissue a homogenous appearance The lesion is clinically often mistaken for a tumour

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NUCLEAR BUDDING AND MICRONUCLEI FORMATION IN HUMAN BONE MARROW, SKIN AND FASCIA LATA CELLS IN VITRO AND IN ORAL MUCOSA CELLS IN VIVO

By

ROBERT N. ELSTON

Received 4 III 63

While lobulated nuclei and fragmented nuclei are a commonly occurring feature of various tumour cell lines (1, 2, 3) the occurrence of such or similar features are not clearly established as a general condition of non pathologic tissue cell lines. *Fogh et al.* (4) in a study on the effects of x-rays on cultured human amnion cells found that irradiation caused budding and break-off of nuclear material, wrinkling and irregularities of the nuclear membrane and vascuolization. *Fogh et al.* do not report upon the condition of control cultures, so we do not know if any of these abnormalities mentioned occur in normal cell strains and with what frequency. The observations reported here would indicate that lobulated nuclei and micronuclei are a common feature of human tissue cells grown in culture and also of the cells of the oral mucosa which did not receive any treatments other than fixation and staining.

Nuclear budding and micronuclei were first observed in tissue culture preparations of human skin, fascia lata and bone marrow which were made for chromosome studies. The cultures were all grown by the tissue culture techniques described by *Böök et al.* (5, 6) and *Fraccaro et al.* (7) and fixed and stained by either the Feulgen or the aceto-orcein method.

A typical nuclear outgrowth or bud is illustrated in Plate 1 a. Nuclear buds and micronuclei are shown in Plate 1 b and in Figs. 1 a, b, c, d. Cells showing the sequence of formation of the micronuclei from the nuclear buds are shown in Figs. 1 a, c, d and e. Only the nuclei are outlined in Fig. 1.

A total of 3 000 nuclei were studied in detail and some three per cent were found to have either a nuclear bud, a micronucleus or both. In some of the preparations up to five per cent of the cells contained these nuclear aberrations. Of some further two hundred preparations studied none were entirely without these features of nuclear bud or micronucleus.



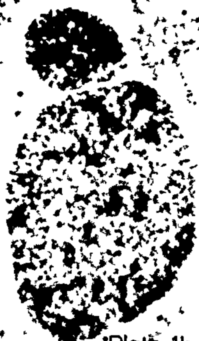




Plate 1a Fascia lata cell in tissue culture Feulgen stained showing nuclear bud containing chromatin material

Plate 1b Skin cell nucleus Feulgen stained tissue culture preparation Showing nuclear bud just prior to separation from the parent nucleus

Plate 1c Oral mucosa cells showing two nuclei Aceto orcein stained One nucleus shows a nuclear outgrowth (Note also the double staining band running medianly around both nuclei This was observed in many cells and is not previously described in the literature)

Oral mucosa cells were scraped from the inner side of the cheek fixed in 95 per cent alcohol and stained directly with aceto orcein. A study of 200 cells gave 6 with 2 doubtful nuclear buds while no true micronuclei were found. Such a typical nuclear bud from an oral mucosa cell is shown in Plate 1 c. Estimation of nuclear budding in these cells is complicated by the pyknotic nature of many of the nuclei and by the possible confusion with polymorphnuclears.

As the patients from whom these biopsies were taken were originally investigated for possible chromosomal abnormalities, the oral mucosa being taken from a normal healthy individual, it would seem that these nuclear features are common to non pathologic tissue cultured cells. Their occurrence in non cultured oral mucosa cells shows that it is unlikely that they are merely cultural artefacts. However, this does not exclude the possibility that other preparative treatments could have effected the formation of the buds. *Zollinger* (8) has shown that even distilled water has a powerful effect on the nuclear structures.

Extrusion of nucleolar material into the cytoplasm of cells in tissue culture has been described by *Lewis* (9) and by *Ludford* (10). They observed the process in living material and did not regard it as abnormal. It is however unlikely that all the structures observed here which lead to the formation of micronuclei containing chromatin material (see Feulgen stained preparations Fig. 1 and Plates 1 a, b) have been confused with similar structures to those observed by *Lewis* and *Ludford* (see however Fig. 1 c).

In the showing of a film on mitosis in endosperm (*Bajer, A. & Mole Bayer, J.* (11)) it was observed that similar protusions to those described here occurred in the C-mitosis. This observation and those of *Atsumi* (1) who observed the formation of budding and lobulation in living tumour ascites under phase contrast give positive evidence that these structures are formed in the living state in a wide variety of tissues both of plant and animal origin and are unlikely to be only cultural artefacts.

Nuclear budding leading to the formation of micronuclei could explain the rare occurrence of hypodiploid cells rang[illegible]

[illegible] micronucleus enter metaphase out of phase then it is conceivable that when the cell is fixed a metaphase plate will be formed with a hypodiploid chromosome number. The micronucleus remaining for example in prophase would not be [illegible]

[illegible] this means we can adequately explain the appearance of hypodiploid chromosome counts where the cell appears intact.

The factors causing the formation of nuclear buds and micronuclei

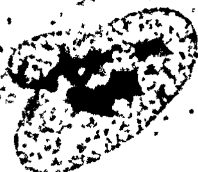






Plate 1a Fascia lata cell in tissue culture Feulgen stained showing nuclear bud containing chromatin material

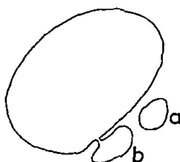
Plate 1b Skin cell nucleus Feulgen stained tissue culture preparation Showing nuclear bud just prior to separation from the parent nucleus

Plate 1c Oral mucosa cells showing two nuclei Aceto orcein stained One nucleus shows a nuclear outgrowth (Note also the double staining band running medianly around both nuclei This was observed in many cells and is not previously described in the literature)

are probably many, and varied They could be a sort of nuclear reaction to various stress conditions of a very different origin and nature We have evidence that they are usually found in cancerous tissuee (1, 2, 14, 15), are increased by irradiation (4) and that oestrone and testosterone affect the freequency of polynucleated cells in a hamster ascites tumour (3) Together with the evidence presented here that nuclear buds and micronuclei are common in the cells of several tissues of human origin of a non pathological nature, both those in tissue culture and those taken directly from the body, there is the possibility of using these nuclear irregularities as a tool to further understand nuclear proliferation and tumour formation

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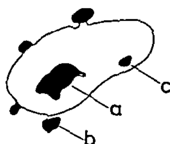




Fig 1a Skin cell nucleus Tissue culture preparation aceto orcein stained a micronucleus budded off and free from parent nucleus b micronucleus just before budding off is completed

Fig 1b Skin cell nucleus Tissue culture preparation feulgen stained Showing micronucleus budding off and containing deeply staining chromatin material

Fig 1c Bone marrow cell nucleus Tissue culture preparation aceto orcein stained Various stages of formation of micronuclei by nuclear budding It would appear that in this case the nucleolus (a) is the origin of the material contained in the buds and the microbody (b) At (c) we may interpret this densely staining body as a nucleolar fragment on the way out This view is based upon the fact that both the nucleolus and the numerous buds stain with approximately the same degree of intensity This is the only case in which such an observation was possible

Fig 1d Bone marrow cell nucleus Tissue culture preparation aceto orcein stained Showing nuclear budding and formation of a micronucleus

Fig 1e Fascia lata cell nucleus Tissue culture preparation aceto orcein stained Showing multiple budding at (a)

TABLE 1

Biochemical Reactions of the Strains

No of strains	Gluc	Rhamnose	Gelatin	Indol	Haemolysis	Oxydase	Nitrate	Mannheim & Stenzel classification
19 strains B anitratum	+	—	—	—	—	—	—	group 1
8 strains B anitratum	+	—	+	—	+	—	—	group 2
1 strain B anitratum	+	+	—	—	—	—	—	group 6
6 strains M lwoffi	—	—	—	—	—	—	—	group 5
3 strains A haemolys	—	—	+	—	+	—	—	group 3
2 strains A haemolys	—	—	—	—	+	—	—	group 3
5 strains M nonliquef	—	—	—	—	—	+	+	group 7

Only the key-reactions are referred to in the table

MATERIAL AND METHODS

Lautrop sent [illegible]
BSW str [illegible]
tratum [illegible]
In ou[illegible] um anitratum 6 strains of Moraxella lwoffi 5 strains of Alkaligenes haemolysans and 5 strains of Moraxella nonliquefaciens

In total the material studied consisted of 28 strains of Bacterium anitratum 6 strains of Moraxella lwoffi 5 strains of Alkaligenes haemolysans and 5 strains of Moraxella nonliquefaciens

All the strains were subject to extensive morphological and biochemical examination so as to verify their identities (Table 1)

The observations were performed on cultures growing on thin (2 4 mm) agar plates which were examined under the high power dry lens of a phase contrast microscope The agar consisted of tryptone (Difco) 0 05 per cent yeast extract 0 05 per cent beef extract 0 02 per cent, sodium acetate 0 02 per cent and agar (Difco) 0 9 per cent adjusted to pH 7 2

The strains were plated out from fresh 24 hour blood agar cultures clockwise around the agar in radiating strokes and incubated at 29 degrees centigrade

The margins of the strokes were then observed at hourly intervals by phase contrast microscopy under the high power dry lens

All the strains were also examined for signs of motility in fluid culture

RESULTS

In observing the margins of the strokes one could generally discern two different kinds of cell arrangement:

1 The cells lying closely together either parallel to the border or at an angle to it, or in closely wound spirals, giving a very regular and "dead" impression

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GLIDING MOTILITY IN THE ORGANISMS BACTERIUM ANITRATUM (B5W), MORAXELLA LWOFFI AND ALKALIGENES HAEMOLYSANS, AS COMPARED TO MORAXELLA NONLIQUEFACIENS

By

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Received 9.iii.63

These organisms belong to a group of gramnegative bacilli, often occurring in diploform, which are strictly aerobic and grow well on simple media.

B5W was first described by *Stuart & van Stratum* in 1945, and again in 1949.

In 1948 *Schaub & Hauber* described a similar organism and gave it the name Bacterium anitratum. Later workers have proved them to be the same organism. Since that time a variety of proposals as to its nomenclature and classification have been forwarded, and there is still no generally accepted opinion on these matters. Moraxella lwoffi was first described by *Audureau* in 1940. As the name indicates it was considered to belong to the genus Moraxella. Alkaligenes (Achromobacter) haemolysans was first described by *Henriksen* in 1936. Its most distinguishing characteristic is a strong, sharply limited, punched out haemolysis on blood agar.

In 1961 *Lautrop* proposed that Bacterium anitratum and Moraxella lwoffi should be transferred to the genus Cytophaga on the basis of their exhibiting gliding motility, which is a characteristic of Myxobacteriales.

In an extensive work published in 1962 comprising 130 strains, *Mannheim & Stenzel* divide these organisms into seven groups based on their biochemical properties.

The present study was undertaken to verify *Lautrop's* results, and if possible, to find other microbes exhibiting the same sort of gliding motility. It would appear to be of special interest to investigate the genus Moraxella for gliding motility.

DISCUSSION

Lautrop's results were verified in full, and our studies showed an even higher frequency of gliding motility among Bacterium anitratum than did his.

Besides our investigations revealed that Alkaligenes haemolysans showed the same kind of gliding motility as did Bacterium anitratum and Moraxella lwoffi. On the basis of these findings we suggest that Alkaligenes haemolysans belongs to the same group of organisms as do Bacterium anitratum and Moraxella lwoffi. This suggestion is also supported by the morphological and biochemical similarities among these microbes.

The 5 strains of Moraxella nonliquefaciens studied did not show gliding motility. Of course, our observations here are too few, but they do suggest that Moraxella lwoffi is not a true Moraxella.

The mechanism of the gliding is not understood.

Electronphotomicrographs show that the organisms are capsulate and that they do not possess flagellae or fimbriae to account for the movements. The fact that the individual cells often glide until they come into contact with other cells where they come to rest, might suggest some sort of chemotaxis.

In a paper which appeared after the completion of this study, *Piechaud* reports finding gliding motility in all Moraxella species, including nonliquefaciens. Further studies are necessary in order to clarify the reason for this discrepancy.

CONCLUSION

Our results support the findings of *Lautrop*. In addition the same sort of gliding motility was found in Alkaligenes haemolysans, and we therefore suggest that also this organism should be transferred to the genus Cytophaga.

According to our findings, the removal of the organism described by *Audureau* in 1940 from the genus Moraxella might seem to be reasonable.

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2. The margins were broken up either by the cells forming spear heads or by their lying haphazardly around, giving a lacelike pattern of cell arrangement.

The lastmentioned type of *arrangement* was *then examined closer* for signs of gliding motility.

In a personal communication *Lautrop* has given a description of gliding motility, to which there is little to add.

Often very few cells glide at a time, and when they move, it is usually very slowly and for very short distances. The distance covered is often not more than one or two cellengths and the speed in the order of mangnitude of 1–10 μ per minute. Movement often starts with a soft jerk and then proceeds for a short while. A change in direction is frequent, and reversal of movement with or without an interposed period of rest is not uncommon. *Most frequently* one sees *isolated cells move* and usually the cell will glide towards other cells and come to rest when it touches them.

The movements often start after 4–5 hours of incubation at 29 degrees centigrade. There are wide variations to this general description of gliding motility, both in number of cells gliding at a time, in speed of movement and in distance covered.

From our observations there seems to be no reason for calling one strain "a very active glider" and another strain "a weak glider". Frequent observations of one strain often revealed that at one time it showed very active movements, and that at a later date the movements would be rather inconspicuous. Only a few of the Bacterium anitratum and Moraxella lwoffi strains examined did not glide at any time of observation (Table 2).

TABLE 2
Results of Tests for Motility

	No. of strains examined	No. of strains gliding	No. of strains not gliding
B. anitratum	28	25 (89.2 %)	3
M. lwoffi	6	4 (66.7 %)	2
A. haemolysans	5	5 (100 %)	0
M. nonliquefaciens	5	0 (0 %)	5

As the table shows 89.2 per cent of the Bacterium anitratum strains showed gliding motility at some time of observation.

66.7 per cent of the Moraxalla lwoffi strains and 100 per cent of the Alkaligenes haemolysans strains exhibited gliding motility.

None of the Moraxella nonliquefaciens strains showed any sign of gliding motility. None of the strains moved *in fluid medium*.

FROM THE UNIVERSITY OF BERGEN SCHOOL OF MEDICINE THE GADE INSTITUTE DEPARTMENT OF MICROBIOLOGY BERGEN NORWAY

SEROLOGICAL TYPING OF *STAPHYLOCOCCUS AUREUS*

1 *Factor a Serum*

By

GUNNAR HAUKENES

Received 25 ii 63

Oeding's method for serological typing of *Staphylococcus aureus* (*Oeding* 1952 and 1957) has now been used for years in many laboratories and has proved to be of considerable value in epidemiological work (cf the review given by *Oeding* 1960)

In a previous paper (*Haukenes & Oeding* 1960) the results of a more thorough study of the antibody composition of one of the factor sera the *e* serum have been reported Two new antigens were discovered and named *m* and *n* Antibodies to *m* were found in other factor sera as well It was therefore decided to examine all the other factor sera by the same technique A preliminary report has recently been given (*Haukenes* 1962), and the results will now be published in detail in a series of articles

As a number of new antigens was detected it soon appeared that the designation system required revision and the new antigens [illegible] [illegible] and correspondingly for new antigens reacting in other sera

MATERIALS AND METHODS

Strains In addition [illegible] [illegible] and agglutination reactions have been [illegible] unless otherwise stated

EXPERIMENTAL AND RESULTS

Factor *a* serum is prepared from antiserum strain 3647 by absorption with strain F 21 In the present work two *a* sera prepared from different 3647 antisera have been compared The agglutination titre of each type strain was estimated in both sera (Table 1) applying serum dilutions of 1 10 1 25 1 50 1 100 1 250, etc

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is not known owing to other strong agglutinogens in these strains and this had to be checked in a pure a_1 serum (see below)

Likewise strains 365 and Cowan I cross reacted and cross-absorbed completely, the common agglutinogen being designated a_2 Also strain 17 A possessed this agglutinogen, but removal of a_2 antibodies left another weak agglutinin for this strain (cf a, below), In addition strains 3647, 2095, Cowan III and 830 absorbed a_2 antibodies, but their reactivity is not known

When the a serum was absorbed with strain 3189, no other influence on the agglutinability of the type strains was observed than the removal of agglutinins to this strain The corresponding antigen has been designated a_3 The a_3 agglutinins were also absorbed by strains 3647, 2095, Cowan III and 830, but it is not known whether these strains were agglutinated by a_3 agglutinins

Strains 3647, 2095, and Cowan III absorbed the two a sera completely, and ...
other ...
in th...
sera might also explain it Further absorptions of the a serum were there-

...gen was designated a_4 The a_4 antibodies were absorbed by none of the other type strains except 830, which, however, did not agglutinate in the serum

Oeding (1953 a) reported that some strains reacted in the a serum also after they had been autoclaved As seen from Table 1 the strains 3647, 17 A, 2095, Cowan III and 830 agglutinated in both a sera *after* autoclaving of the organisms at 120° C for 2 hours This is in accordance with *Oeding*'s observations, strain 830 though not included in his studies The same 5 strains were also found to absorb all antibodies to autoclaved bacteria, even without autoclaving the organisms used for absorption, while none of the other strains were able to absorb these antibodies The heat stable antigen has been designated a , and is not identical to any of the other above-mentioned antigens since absorption with strain 17 A removed only a_2 of the other antibodies, and the other two a_2 strains, 365 and Cowan I, were unable to absorb a antibodies

Preparation of Individual Factor Sera

Factor a_1 serum As reported above strain 830 possesses antigens a_2, a_3, a_4, and a_5, and the original a serum was therefore absorbed with this strain for the preparation of an a_1 serum The resulting serum agglutinated strains 1503 2253, and 28, very weakly also strain 3647, but none of the other a_1 strains (Table 3)

TABLE 1

Agglutinability of the Type Strains in Two a Factor Sera

Strain	a factor serum K 827		a factor serum K 828	
	Live bacteria	Autoclaved bacteria	Live bacteria	Autoclaved bacteria
1503	++ (25)	—	(+)	—
2253	++ (25)	—	(+)	—
28	++ (25)	—	(+)	—
365	+++ (50)	—	(+)	—
3647	+++ (1000)	++	+++ (250)	+(+)
17 A	++ (50)	++	++ (10)	++
3189	++ (100)	—	++ (50)	—
2095	+++ (500)	++	+++ (250)	++(+)
Cowan I	++ (100)	—	+ (10)	—
Cowan III	+++ (500)	++	+++ (250)	++
830	+++ (100)	++(+)	+++ (100)	++
F 21, Wood 46 and Cowan II	—		—	—

(+), + ++ and +++ Strength of agglutination
— No agglutination in dilution 1 in 10
Figures in brackets Resiprocals of agglutination titres

TABLE 2

Different Patterns of Agglutination after Absorption of a Factor Serum K 827 with Type Strains

Strain used for agglutination	Serum a K 827 absorbed with strain					Un absorbed
	F 21	1503	365	3647	3189	
1503	++	—	++		++	++
365	++(+)	++	—		++	+++
3647	+++	+++	+++	—	+++	+++
3189	++	++	++	—		++

Code See Table 1

Table 1 shows that *a* factor serum K 827 is more potent than K 828 To get more information of their antibody composition both factor sera were absorbed with each of the 14 type strains The growth of one Roux bottle was added to one ml of the serum, *i e* thrice the dose of bacteria ordinarily used for preparation of the factor sera

Table 2 shows the agglutinability of some type strains in some of the absorbed sera Only one representative of the strains showing identical agglutinability and absorbing capacity has been included Four different patterns of reaction were obtained indicating at least three different antibodies

The strains 1503, 2253, and 28 showed complete cross-agglutination and cross absorption, having thus a common agglutinogen which is designated a_1 Antibodies to a_1 were also removed by strains 3647, 2095 Wood 46, and Cowan III, Strain Wood 46 did not agglutinate in the *a* serum, cf Table 1 The reactivity of strains 3647, 2095 and Cowan III

is not known owing to other strong agglutinogens in these strains and this had to be checked in a pure a_1 serum (see below)

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Strains 3647, 2095, and Cowan III absorbed the two a sera completely, and these strains agglutinated strongly in all sera absorbed with the other strains except 830 This might be due to a special shared antigen in these strains, but a strong reactivity in each of the other three factor sera might also explain it Further absorptions of the a serum were therefore performed with strains 1503, 17 A and 3189 successively, thus removing the other known antibodies (including a_5 antibodies, cf below) In this serum the strains still agglutinated strongly, and the new agglutinogen was designated a_4 The a_4 antibodies were absorbed by none of the other type strains except 830, which, however, did not agglutinate in the serum

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3189	++ (100)	—	++ (50)	—
2095	+++ (500)	++	+++ (250)	++(+)
Cowan I	++ (100)	—	+ (10)	—
Cowan III	+++ (500)	++	+++ (250)	++
830	+++ (100)	++(+)	+++ (100)	++
F 21, Wood 46 and Cowan II	—		—	—

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365	++(+)	++			++	+++
3647	+++	+++	+++		+++	+++
3189	++	++	++			++

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TABLE 5

The Antigenic Formula of 18 Type Strains with Regard to the Different a Factors

Strain	Antigenic formula				
1503	a_1				
2253	a_1				
28	a_1				
365		a_2			
3647	a_1	a_2*	a_3*	a_4	a_5
17 A		a_2			a_5
3189			a_3		
2095	a_1	a_2*	a_3*	a_4	a_5
Wood 46	(a_1)				
Cowan I		a_2			
Cowan III	a_1	a_2*	a_3*	a_4	a_5
670		(a_2)	(a_3)		
830		a_2*	a_3*	(a_4)	a_5
5687	(a_1)	(a_2)	(a_3)	(a_4)	(a_5)
6376	(a_1)	(a_2)			(a_5)

( ) The strain absorbs antibodies but does not agglutinate
* *The strain absorbs the antibodies but its agglutinability is not known*
Two strains Cowan II and 1015 have not been included in the table as they possess none of the a antigens

the other a_2 strains contained demonstrable a_2 antibodies It has not *been possible to prepare a pure a_2 factor* serum from serum 3647 owing to the presence of a_1 antibodies, as we have at present no strain possessing the a_1 but not the a antigen

Factor a_3 serum Both 3647, 2095, and Cowan III antisera contained *good* a_3 antibodies As for the preparation of an a_3 serum from these sera, *the* same difficulties were met with as mentioned above in connection with the a_2 serum Serum 3189 possessed only weak agglutinins to the homologous strain after absorption with *strain F 21, and the* presence, if any, of f and g antibodies has also to be taken into account (4)

Factor a_4 serum As described above this serum can be prepared by absorption of serum 3647 with strains F 21, 17 *A*, *3189*, and *1503* successively *The agglutinability* of the type strains in an a_4 serum is shown in Table 4 Strong a_4 antibodies were also found in other 3647 sera and in 2095 and Cowan III *antisera as well*

Factor a_5 serum Neither this serum can be prepared from 3647, 2095, Cowan III or 830 antisera owing to their content of a_1 antibodies An a_5 factor serum has been prepared from serum 17 A by absorption with strain 1503 and a new *strain, 670 This will* be discussed in a forthcoming paper in connection with the h factors

Distribution of the a Antigens among the Type Strains

In Table 5 the antigenic composition of the type strains with regard to the a factors has been *given* Four *other* strains have also been pre-

TABLE 3

Agglutinability of the Type Strains in a_1 Serum
(Serum 3647 K 827 Absorbed with Strains F 21 and 830)

Strain	a_1 serum
1503	++
2253	+(+)
28	++
3647	(+)
The other type strains	—

Code See Table 1

TABLE 4

Agglutinability of the Type Strains in an a_1 Factor Serum
(Serum 3647 K 827 Absorbed with Strains F 21 1503 17 A and 3189)

Strain	a_1 serum
3647	++(+) (100)
2095	++ (100)
Cowan III	++(+) (100)
830	(+) ?

Code See Table 1

Two more 3647 antisera were examined, but they contained lesser amounts of a_1 antibodies than the serum used in these experiments (K 827)

The immune sera of the other a_1 strains were also examined for a_1 antibodies Sera 2095 and Wood 46 did not contain a_1 antibodies in detectable amounts Serum Cowan III appeared to have rather strong a_1 antibodies, but also strong n antibodies which agglutinate the same strains We have at present no strain available for removal of the interfering n antibodies from this serum without removing a_1 antibodies as well, as strain 830 is not in the possession of the n antigen The same reservation has to be made regarding preparation of an a_1 serum from serum 3647 which may contain small amounts of n antibodies (2) Before a 3647 serum is used for this purpose it should therefore always be checked for the presence of n antibodies by absorption with strain 2095 which will remove a_1 but not n antibodies If no agglutination of strain 1503 is obtained after such absorption, no interference from n antibodies is likely to occur None of our 3647 sera were found to have n agglutinins in detectable amounts Whether the 1503 2253 and 28 immune sera contain a_1 antibodies has not been settled owing to their content of e and n antibodies which cannot be removed without removing also a_1 antibodies Strain 365, which according to previously published results (2) absorbs e and n antibodies failed to do so when re examined

Factor a_2 serum Of four 3647 immune sera examined only one (K 827) yielded good titre of a_2 antibodies None of the sera against

tions to be reported in the next article have shown that the agglutination of strain 3189 in the *b* serum is due to a_3 antibodies One might therefore conclude that the 3647 serum used by *Oeding* for some reason has not contained a_3 antibodies

Considering the varying antibody compositions of the different 3647 sera in the present investigations and comparing with *Oeding's* results, it is to be expected that more new antibodies will be detected in future It should therefore be emphasized that every serum has to be controlled by various absorptions before being used for production of factor sera

Only the a_5 antigen appears to be heat-stable This is in accordance with the results reported by *Oeding* (5), who found that neither autoclaving nor treatment with trypsin destroyed the agglutinogens of strains 3647, 2095 17 A and Cowan III, the same strains which were shown to possess the a_5 antigen in the present investigations The a_5 antigen will be discussed in more detail in connection with the *h* antigens

The *a* factor serum prepared by the standard method is too complex to be of value in serological typing of staphylococci The distribution of the various *a* factors among pyogenic staphylococci is at present not known and accordingly not their significance for the purpose of classification Strains 3647, 2095, and Cowan III contain all five *a* antigens and were the only strains which agglutinated in the a_1 serum The a_1 antigen seems to be the major antigen of these strains which all belong to phage group III with almost identical phage patterns (*Oeding* 1953b) It is therefore of special interest to find out whether the a_1 antigen is representative for this phage group Investigations on this subject are in progress in this laboratory (3)

SUMMARY

Factor *a* serum (*Oeding* 1952) was shown to be composed of antibodies to five different antigens, preliminarily designated a_1, a_2, a_3, a_4, and a_5 Only the a_5 antigen was found to be heat-stable Individual factor sera to a_1, a_4, and a_5 have been prepared

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3 *Hofst*

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sented, since these strains will be included among the type strains for preparation of other factor sera which will be discussed in forthcoming papers

With our present set of type strains the following factor sera may be prepared:

a_1 serum: Absorption of serum 3647 with strains F 21 and 830 after checking for n antibodies

$a_2 a_1$ serum: Absorption of serum 3647 with strains F 21, 1503, 3189, and autoclaved 3647

$a_3 a_1$ serum: Absorption of serum 3647 with strains F 21, 1503, and 17 A

a_4 serum Absorption of serum 3647 with strains F 21, 1503, 17 A, and 3189

a_5 serum: Absorption of serum 17 A with strains 1503 and 670

DISCUSSION

The presence of several antibodies in the a serum was suspected for some time, as different agglutination patterns were observed in a factor sera originating from different strain 3647 immune sera In the present experiments antibodies to at least five different antigens have been revealed It has also been shown that the heat-stable a_5 antigen described by *Oeding* (5), can be separated from the other antigens which are heat-labile

Oeding (1952) has pointed out that the a factor serum should be prepared by careful absorption with strain F 21, as this strain also possessed a weak a antigen As a consequence his a sera frequently agglutinated strain F 21 weakly and were rather poor in agglutinin content All strains were therefore in addition tested in a more potent a serum, which, however, also possessed c antibodies, the ac factor serum This difficulty was not met with in the present investigations, as absorption of the a serum with thrice the standard dose of F 21 bacteria led to no decrease in the agglutination titres It is probable that antibodies to another 3647 antigen has been present in *Oedings's* a sera Investigations which will be reported in a later article, have brought evidence that this disagreement is caused by c antibodies The c antigen is partly blocked in strain F 21, and inadequate absorption with this strain might leave c antibodies

It was surprising to find that strain 3189 agglutinated in the a factor serum, as this strain was the only one among the original type strains which was claimed to lack the a antigen and was therefore used for the preparation of ac serum Most probably this discrepancies is due to different antibody composition of the 3647 immune sera On the other hand strain 3189 has previously been found to have a b antigen The b serum is prepared from serum 2095 by absorption with strain 2253 Investiga-

FROM THE UNIVERSITY OF BERGEN SCHOOL OF MEDICINE THE GADE INSTITUTE DEPARTMENT OF MICROBIOLOGY BERGEN, NORWAY

SEROLOGICAL TYPING OF *STAPHYLOCOCCUS AUREUS*

2 *Factor* b *Serum*

By

GUNNAR HAUKENES

Received 1 iii 63

In a previous paper (2) 5 different antibodies were found to constitute the *a* factor serum Two different whole sera, which were analysed with regard to content of the different *a* antibodies, varied considerably

Oeding (1952) found agglutinins to a heat stable *b* antigen and *Grun* (1962) has suggested that the antigen is identical to the heat stable antigen of strain 17 A which reacted in the *h* serum Investigations of the *a* antigens brought evidence that this heat stable antigen is the a_5 antigen (2)

The *b* factor serum is prepared from serum 2095 and has been subjected to the same analyses as the *a* serum (2) One of the 3647 sera which were used for preparation of the *a* factor serum, was also analysed for *b* antibodies for comparison and to facilitate the identification of the agglutinins

MATERIALS AND METHODS

Strains In addition to the 1[illegible] two following strains hav[illegible] 1015 (W Kretschmar Ba[illegible] by the routine set of 24 r[illegible]

EXPERIMENTAL AND RESULTS

The *b* serum is prepared by absorption of serum 2095 with strain 2253 (4) Apart from lacking the *n* antigen strain 2095 has been found to have the same antigenic structure as strain 3647, including all of the *a* antigens Antibodies to the latter antigens might therefore be expected to be present in a 2095 serum Absorption with strain 2253 removes a_1 antibodies and according to *Oeding* (4) also *c* antibodies

The agglutinability of the type strains in the *b* serum is shown in Table 1 Absorption of serum 3647 with strain 2253 should theoretically

6 *Oeding P* Serological typing of staphylococci III Further investigations and comparison to phage typing Acta path et microbiol scandinav 33 324 336 1953b

7 *Oeding P* Agglutinability of pyogenic staphylococci at various conditions Acta path et microbiol scandinav *41* 310 324 1957

8 *Oeding P* Antigenic properties of Staphylococcus aureus Bact Rev 24 374 396 1960

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Oeding (1952) found agglutinins to a heat stable b antigen and Grün (1962) has suggested that the antigen is identical to the heat stable antigen of strain 17 A which reacted in the *h* serum Investigations of the *a* antigens brought evidence that this heat-stable antigen is the a_3 antigen (2)

The *b* factor serum is prepared from serum 2095 and has been subjected to the same analyses as the *a* serum (2) One of the 3647 sera which were used for preparation of the *a* factor serum, was also analysed for *b* antibodies for comparison and to facilitate the identification of the agglutinins

MATERIALS AND METHODS

Strains In add tion to the 14
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1015 (B *Kret schmar*
by the routine set of 2
Lab ratory Service Co
antigens a_1 a_2 and a_3

The antisera were diluted 1 10 1 25 1 50 1 100 1 250 etc for the estimation f the agglutination titres Immunization agglutinin absorption and agglutination were performed by the technique described by *Oeding* (1957)

EXPERIMENTAL AND RESULTS

The *b* serum is prepared by absorption of serum 2095 with strain 2253 (4) Apart from lacking the *a* antigen strain 2095 has been found to have the same antigenic structure as strain 3647, including all of the *a* antigens Antibodies to the latter antigens might therefore be expected to be present in a 2095 serum Absorption with strain 2253 removes a_1 antibodies and according to *Oeding* (4) also *c* antibodies

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8 *Oeding P* Antigenic properties of Staphylococcus aureus Bact Rev 24 374-396 1960

TABLE 2

Agglutinability in b Factor Serum after Absorption with some Type Strains

Agglutinating strain	*b* serum* absorbed with								Unabsorbed
	28	365	F21	17A	3189	2095	Cowan III	830	
28	—	—	—	+	(+)	—	—	+	++
365	++	—	—	++	++	—	—	—	+++
3647	+++	+++	+++	+++	+++	—	—	+	+++
F 21	++	—	—	++	++	—	—	+	+++
17 A	—	+	+	—	—	—	+	—	++
3189	++	+	+	++	—	—	—	—	++
2095	+++	+++	+++	+++	+++	—	(+)*	+	+++
Cowan III	+++	+++	+++	+++	+++	—	—	(+)	+++

Code See Table 1
* Serum 2095 absorbed with strain 2253

tinins which have been designated b_3 28, 3647, F 21, 17 A, 3189, 2095, Cowan II, 830, and 6376

Strain Cowan I agglutinated weakly, but the agglutination was not to a_3 antibodies, since the agglutinins were removed by absorption with strain 1503 The following strains seemed to absorb the agglutinins to Cowan I 1503, 28, 365, 3647, F 21, (17A?), 2095, Cowan II, Cowan III, 830, and 6376 This antigen has preliminarily been designated b_4, but the agglutination reactions in the absorbed sera were too weak to be relied upon for classification

The *b* agglutinins against strain Cowan II were also weak and were absorbed by strains 3647, 2095, Cowan II, Cowan III, 830, and 6376 The corresponding antigen will preliminarily be given the designation b_5 The same reservation, however, has to be made with regard to the reliability of the results as stated for the b_4 antigen The b_4 and b_5 antigens will not be included in the antigenic formulas of the type strains, until the results have been controlled in more potent sera

Oeding (4) preferred to prepare the *b* serum from serum 2095 and not from serum 3647 which is used for the *a*, *ac*, and *c* sera, because serum 2095 gave a stronger *b* serum Considering the "*b*" serum from 3647 in Table 1 it is apparent that this serum contains at least one antibody more than the *b* serum from 2095, represented by the strong agglutination reactions obtained with strains 1015 and 6376 Absorption of the "*b* serum with strain 2095 did not remove these ... and the ...

2

f ... sera As a consequence *b* factor serum cannot be prepared from serum 3647 unless absorption is also performed with strain 1015

The antibody composition of the *b* factor serum analysed here is a_1, a_4, a_5, b_1, b_2, and b_3 No a_2 antibodies were demonstrated, but they may possibly be found in other 2095 sera

TABLE 1

Agglutinability of the Type Strains in b *Factor Serum (Serum 2095 Absorbed with Strain 2253), and in a "b" Serum Prepared from Serum 3647*

Strain	*b* serum		"*b*" serum	
	Live bacteria	Autoclaved bacteria	Live bacteria	Autoclaved bacteria
28	++ (100)	—	—	—
365	+++ (100)	—	+++ (250)	—
3647	+++ (500)	++	+++ (500)	±
F 21	+++ (250)	—	+++ (500)	++
17 A	++ (10)	+++	++ (10)	++
3189	++ (100)	—	++ (50)	—
2095	+++ (500)	+++	+++ (1000)	+++
Cowan I	+ (10)	—	+ (10)	—
Cowan II	+ (10)	(+)?	—	—
Cowan III	+++ (500)	++	+++ (250)	++
1015	—	—	+++ (100)	+(+)
830	++ (100)	+++	+++ (250)	+++
6376	—	—	++ (100)	++

Code (+), +, ++, and +++ denote strength of agglutination
Figures in brackets Resiprocals of agglutination titres

Strains 1503, 2253, and Wood 46 were not agglutinated and have not been included in the table

also yield a *b* serum Of the two 3647 sera analysed for *a* antibodies (2), only serum 3647 K 828 possessed specific *b* antibodies This '*b*' serum has also been presented in Table 1 for comparison

It is at once apparent from the agglutination reactions in the *b* serum (Table 1) that at least one specific *b* antibody is present The agglutination of strain 28 cannot be due to a_1 agglutinins, and the strong 365 agglutination cannot be due to a_2 antibodies since strain Cowan I was only weakly agglutinated Moreover, strain F 21 which possessed no *a* antigens, reacted strongly After autoclaving of the bacteria the same strains reacted as in the *a* serum, which was to be expected

To get more information of the antibodies of this serum, portions were absorbed with each of the type strains in a proportion of 1 ml serum to the growth of 1 Roux bottle of the absorbing strain The various patterns of agglutination with regard to *b* antibodies have been given in Table 2 It appears that a strong agglutinogen is shared by strains 365 and F 21, and the corresponding antibodies were in addition absorbed by strains 2095, 3647, Cowan III, 830 and 6376 This antigen appears to be the major *b* antigen and has been designated b_1

Strain 28 did not absorb b_1 agglutinins, and the agglutination of strain 28 must be due to another *b* antibody, designated b_2, being also absorbed by the strains 1503, 365, 3647, F 21, 2095, Cowan III, 6376 and possibly 3189

Absorption of the *b* serum with strain Cowan III left residual agglutinins for strain 17 A The 17 A agglutination was weak in most of the absorbed sera, and the following strains seemed to absorb these agglu-

b_1 serum The k antigen is heat stable, while the b_1 antigen is heat-labile, and after absorption with autoclaved 365 organisms an apparently pure, but rather weak b_1 serum ensued

The 6376 and 830 antisera did not contain b_1 antibodies in detectable amounts

Factor b_2 serum Strain 830 possesses the antigens a_2, a_3, a_4, a_5, b_1, b_3, b_4, and b_5, and will leave only b_2 antibodies when used for absorption of the b serum A b_2 serum prepared in this way is shown in Table 2 where rather weak agglutinations were obtained Attempts to prepare a b_2 serum from sera 1503 and 28 by absorption with strain 2253, and from sera 3647, Cowan III and F 21 by absorption with strains 2253 and 830 were not successful None of the sera 365, 3189 and 6376 contained demonstrable b_2 antibodies

Factor b_3 serum As shown in Table 2 this serum can be prepared by absorbing serum 2095 with strain Cowan III A somewhat weaker b_3 serum was produced from serum 3647 Whether b_3 agglutinins are present in 17 A sera is at present not clear, since the only one of our type strains which removes the strong h agglutinins of serum 17 A, strain 670, removes b_3 antibodies as well b_3 antibodies have not been demonstrated in sera against the other b_3 strains

Factor b_4 and b_5 sera have not been prepared As mentioned above these agglutinins were weak in the sera tested and cannot yet be used for typing purposes Their possible presence in staphylococcal immune sera should, however, be known by workers in this field

TABLE 4

Distribution of the b Antigens Among the Type Strains

Strain 1503		(b_2)		(b_4)	
" 28		b	(b_3)	(b_4)	
" 365	b_1	(b_2)		b_4*	
" 3647	b_1	b_2	(b_3)	b_4*	b_5*
F 21	b_1	(b_2)	(b_3)	b_4*	
" 17 A			b_3	(b_4)?	
" 3189		(b_2)?	(b_3)		
2095	b_1	b_2	b_3	b_4*	b_5*
" Cowan I				b_4	
Cowan II			(b_3)	b_4*	b
" Cowan III	b_1	b_2		b_4*	b_5*
" 830	(b_1)		(b_3)	b_4*	b_5*
" 670			(b_3)	(b_4)	(b_5)
" 5687	(b_1)	(b_2)	(b_3)	(b_4)	(b_5)
" 6376	(b_1)	(b_2)	(b_3)	(b_4)	(b_5)

Code ( ) The strain absorbs the antibodies but does not agglutinate
* The agglutinability of the strain is unknown

Strains 2253 Wood 46 and 1015 possessed no b antigens and have not been included in the table

TABLE 3

*Agglutinability of Type Strains in a 'b1' Serum Before and after Absorption with Strain 2095 **

Strain	b_1 serum	b_1 serum absorbed with strain 2095*
365	++	—
3647	++	—
F 21	+++	+++
2095	++	—
Cowan III	++	—

Code See Table 1

The "b_1" serum was prepared by absorption of serum F 21 with strains 1503 Wood 46, and 1015

* Absorption with the other b_1 strains except F 21 gave the same agglutination pattern

Preparation of Individual Factor Sera

Factor b_1 serum This serum can at present not be prepared from 2095, 3647, or Cowan III antisera, since a_1 agglutinins cannot be removed without removing also b_1 agglutinins Attempts were therefore made to prepare the b_1 serum from serum F 21, which probably contain b_1, b_2, b_3, c_1, ι, and m antibodies Absorption was first carried out with strain Wood 46 to remove ι antibodies, thereafter with strain 1503 to remove b_2 and m antibodies Strain 1015 was used for absorption of c_1 antibodies Since the resulting serum did not agglutinate strain 17 A, absorption for removal of b_3 antibodies was not undertaken The type strains reacted in this serum as shown in Table 3

Since the b_1 serum was produced from a whole serum which has yet not been analysed more thoroughly, its purity was controlled by absorption with each of the agglutinating strains None of the strains except F 21 exhausted completely the b_1 serum An additional absorption with strain Wood 46 was performed to ensure that all ι antibodies had been removed, but without influence on the F 21 agglutination (Table 3)

This new antigen of strain F 21 is more correctly classified as an ι antigen and has been designated ι_2 The $b_1\iota_2$ serum prepared in this way agglutinated the b_1 strains strongly, and is well suited for serological typing when used in combination with an ι_2 serum, until our set of type strains has been extended The ι antigen will be discussed in a forthcoming paper

An attempt was also made to prepare a b_1 serum from serum 365 Strain 365 has been found to possess the antigens a_2, b_1, (b), b_1, (e), (ι), k, m, and (n), but absorption experiments indicated that only b_1, k, and m antibodies were present in our 365 serum Absorption with strain Cowan I removed m antibodies and gave a b_1k serum The agglutination reactions were weaker in this serum than in the serum above, but together with a k serum it may be used as a supplement to the other

composition of the rabbit immune sera and one need not presume a change in the antigenic pattern of the strain

It has previously been observed (2) that the strains which possessed the a_1 antigen, i.e. strains 3647, 2095, Cowan III and 830 had got all or almost all of the other *a* antigens. Considering Table 4 this proved to be true also for the *b* antigens.

No other heat stable antigen than the a_5 antigen was demonstrated in this study. Moreover, no heat stable antigen other than the a_5 antigen has been found to be shared by strains 2095 and 17 A, as will be reported later. The common heat stable antigen of *h* and *b* strains reported by *Grün* (1962) is undoubtedly the a_5 antigen.

Oeding (4) used only 9 type strains in his studies and not the strains 1015 and 6376 which agglutinated strongly in the *b* serum prepared from serum 3647 but not in the actual *b* serum (cf. Table 1). Whether *Oeding* was aware of this is uncertain. It should also be mentioned that strain 2253, which is used for absorption, was claimed to possess the *c* antigen and possibly the *c* agglutinins were removed. In the present experiments strain 2253 did not remove c antibodies and it was first thought that strain 2253 had lost its *c* antigen. Later it has been discovered that the *c* antigen may sometimes be blocked in this strain and can be revealed by cultivation on mannitol salt agar. This will be reported in the forthcoming paper.

SUMMARY

In the present study of the *b* factor serum several new antigens have been detected. It was also found that the *b* serum contained different *a* antibodies, as was expected from previous investigations of the distribution of the *a* antigens.

Some discrepancies with regard to earlier investigations of the *b* serum have been discussed. The varying antibody composition of different immune sera and the occurrence of antigen blocking are apparently important reasons.

When pure factor sera can be prepared and with a revised technique for revealing blocked antigen, reproducible and comparable results will possibly be obtained.

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3. *Ha*[illegible]
4. O[illegible]
5. O[illegible]

Distribution of the b Antigens among the Type Strains

The antigenic structure of the type strains with regard to *b* antigens has been presented in Table 4, including also two strains, 670 and 5687, which will be described in connection with the *h* antigens in a forthcoming article The b_4 and b_5 antigens have also been included, but the reader is referred to the reservations made above

At present the following factor sera can be prepared

b_1 serum Absorption of serum 365 with strain Cowan I and autoclaved 365 organisms Gives a rather weak serum

b_1k serum Absorption of serum 365 with strain Cowan I

b_{1l2} serum Absorption of serum F 21 with strains Wood 46, 1503, and 1015 This serum was found to contain strong b_1 antibodies

b_2 serum Absorption of serum 2095 with strain 830

b_3 serum Absorption of serum 2095 with strain Cowan III

DISCUSSION

Agglutination and cross-absorption experiments have shown that also the *b* factor serum is composed of different antibodies Five new agglutinogens have been detected and have been designated as *b* antigens since they were first detected in the *b* serum This is especially emphasized in this connection to avoid confusion, since *b* agglutinins may possibly be found in *a* sera when prepared from 3647 sera other than those examined in the previous article

The majority of the type strains which agglutinated in the *a* serum agglutinated also in the *b* serum, due to strong common agglutinins The principal difference between the two factor sera is the lack of a_1 agglutinins in the *b* serum and the lack of b_1 and b_2 agglutinins in the *a* serum, while at least a_3, a_4, and a_5 agglutinins are shared by the two sera

As demonstrated previously for the *a* serum the strong agglutinability of strains *3647, 2095, Cowan III and 830 in the b serum also appeared* to be due to the a_4 agglutinins

The b_1 antigen appears from the present and other investigations to be the major *b* antigen Most probably factor b_1 serum will be of great value in serological typing yielding relatively strong and distinct reactions with most b_1 strains No correlation has been found between the b_1 antigen and the bacteriophage groups, since strain 365 was not typable and the remaining b_1 type strains belonged to phage group II or III

As mentioned in the previous article *the agglutination of strain 3189* in the *b* serum is due to a_3 antibodies, and no specific *b* agglutinin to this strain has been detected Strain 3189 was classified by *Oeding* (4) as a *b* strain which possessed no *a* antigen The obvious cause of this *apparent discrepancy* is the frequently observed different antibody

composition of the rabbit immune sera, and one need not presume a change in the antigenic pattern of the strain

It has previously been observed (2) that the strains which possessed the a_4 antigen, i e strains 3647, 2095, Cowan III, and 830, had got all or almost all of the other *a* antigens Considering Table 4 this proved to be true also for the *b* antigens

No other heat stable antigen than the a_5 antigen was demonstrated in this study Moreover, no heat-stable antigen other than the a_5 antigen has been found to be shared by strains 2095 and 17 A, as will be reported later The common heat-stable antigen of *h* and *b* strains reported by *Grun* (1962) is undoubtedly the a_5 antigen

Oeding (4) used only 9 type strains in his studies, and not the strains 1015 and 6376 which agglutinated strongly in the "*b*" serum prepared from serum 3647, but not in the actual *b* serum (cf Table 1) Whether *Oeding* was aware of this is uncertain It should also be mentioned that strain 2253, which is used for absorption, was claimed to possess the *c* antigen, and possibly the *c* agglutinins were removed In the present experiments strain 2253 did not remove *c* antibodies, and it was first thought that strain 2253 had lost its *c* antigen Later it has been discovered that the *c* antigen may sometimes be blocked in this strain and can be revealed by cultivation on mannitol salt agar This will be reported in the forthcoming paper

SUMMARY

In the present study of the *b* factor serum several new antigens have been detected It was also found that the *b* serum contained different *a* antibodies, as was expected from previous investigations of the distribution of the *a* antigens

Some discrepancies with regard to earlier investigations of the *b* serum have been discussed The varying antibody composition of different immune sera and the occurrence of antigen blocking are apparently important reasons

When pure factor sera can be prepared, and with a revised technique for revealing blocked antigen, reproducible and comparable results will possibly be obtained

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FROM THE UNIVERSITY OF BERGEN, SCHOOL OF MEDICINE
THE GADE INSTITUTE DEPARTMENT OF MICROBIOLOGY, BERGEN NORWAY

SEROLOGICAL TYPING OF *STAPHYLOCOCCUS AUREUS*

3 *Factor* ac *and* c *Sera*

By

GUNNAR HAUKENES

Received 28 iii 63

Previously reported investigations of the *a* and *b* factor sera have revealed that the two sera have several common agglutinins, while other agglutinins were specific for each serum (3, 4)

The *ac* and *c* sera are prepared from serum 3647 like the *a* serum, and the presence of several *a* and *b* antibodies can be predicted The main purpose of the present investigations has therefore been to search for specific *ac* and *c* antibodies

New methods for detection of blocked antigens have been studied in connection with investigations of the *h* antigens It was found that when staphylococci are grown on mannitol-salt agar, several blocked heat-stable agglutinogens can be unveiled The method also appeared to be well suited for demonstration of the c_1 antigen in some strains and has been adopted in the present study More details of this new method will, however, be given in the next article

METHODS

Cultivation In addition to cultivation on nutrient agar slants the bacteria were streaked on mannitol salt agar plates and incubated at 37° C for 18 hours The mannitol salt agar medium contains 7 5 per cent sodium chloride 1 per cent mannitol and phenol red as indicator

Otherwise the materials and methods are the same as in earlier publications (3 4)

EXPERIMENTAL AND RESULTS

The *ac* factor serum (5) is prepared from serum 3647 by absorption with strain 3189 and may therefore be expected to contain a_1, a, a_4, a_5, b_1, and possibly b_4 and b_5 antibodies The agglutination reactions of the type strains in two *ac* sera are shown in Table 1

When the two *ac* sera are compared with the two *a* sera which were prepared from the same 3647 sera (Table 1 in (3)), it appears that apart from the agglutination of strain F 21 in *ac* K 827 no significant difference is noted as for the two K 827 factor sera The *ac* K 828 serum, however, differs greatly, yielding rather strong agglutination reactions

TABLE 1

Agglutinability of the Type Strains in Two ac Factor Sera Prepared from Different 3647 Immune Sera

Strain	*ac* K 827		*ac* K 828	
	Live bacteria	Autoclaved bacteria	Live bacteria	Autoclaved bacteria
1503	++(+) (100)	—	(+)	—
2253	++ (100)	—	(+)	—
28	++ (100)	—	(+)	—
365	++(+) (100)	—	+++ (250)	+
3647	+++ (500)	+++	+++ (500)	++
F 21	+ (25)	—	+++ (500)	++(+)
17 A	++ (50)	+++	++ (10)	+++
2095	+++ (500)	+++	+++ (500)	+++
Cowan I	++(+) (100)	—	+ (10)	—
Cowan II	(+)	+	—	—
Cowan III	+++ (500)	+++	+++ (500)	++(+)
1015	+ (10)	—	+++ (250)	++
830	+++ (250)	+++	+++ (250)	++
6376	—	—	+++ (250)	++

Code (+) to +++ Strength

TABLE 2

Absorption Experiments to Distinguish a_1 and ac_1 Agglutinins

Agglutinating strain	Serum 3647 K 827 absorbed with					
	F 21	F 21 and Wood 46	3189	3189 and Wood 46	3189 and 830	3189 830 and Wood 46
1503	++	—	++(+)	++	++	—
2253	++	—	++	++	++	—
28	++	—	++	++	++	—
Residual antibodies	a_1		a_1 ac_1	ac_1	a_1	

Code See Table 1

with strains 365, F 21, 1015, and 6376, with the latter three also after autoclaving

Although the titre differences between the two K 827 sera were not significant, it was repeatedly observed that strains *1503*, *2253*, and *28* agglutinated more strongly in the *ac* serum when compared with the *a* serum on the same slide Absorption of *ac* K 827 serum with each of the type strains *confirmed* this suspicion of a special *ac* antigen, since absorption with *strain* Wood *46*, which removes a_1 agglutinins, did not remove all agglutinins *to strains 1503*, *2253*, and 28 The new antigen is designated ac_1 To reveal the distribution of the ac_1 antigen among the type strains the *ac serum* was first absorbed with strain Wood 46 to

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TABLE 4

Agglutination with Nutrient Agar and Mannitol Salt Agar Cultures in Three c_1 Sera

Strain	Ser 3647 abs with 2095		Ser 1015 abs with 2095		Ser 6376 abs with 2095	
	Nutrient agar	Mannit agar	Nutrient agar	Mannit agar	Nutrient agar	Mannit agar
253	—	+++	—	+++	—	+++
647*	—	+++	—	+++	—	+++
21*	(+)	+++	—	+++	(+)	+++
owan III	+++	+++	++	+++	+++	+++
1015	+++	+++	+++	+++	+++	+++
376	+++	+++	+++	+++	+++	+++

Code * The strains were sometimes agglutinable also with nutrient agar cultures Cf otherwise Table 1

varied with regard to c_1 antibodies Also the agglutinability of these strains varied from one culture to another A weak agglutination with strain 2253 was also occasionally observed The latter observation is of special interest, since strain 2253 is used for preparation of the *b* factor serum as the strain was previously found to absorb *c* antibodies (5) The c_1 antigen is heat-stable, but rather weak reactions were obtained with autoclaved bacteria Thus the c_1 antigen seems to be partially or completely blocked in some strains

Table 3 shows a c_1 factor serum in the column for absorption with strain 2095 Since other heat stable antigens are readily demonstrated when mannitol salt agar cultures are used for agglutination, this method was attempted with the c_1 factor serum It appears from Table 4, which shows the agglutinability of the type strains in three c_1 sera, that this new method yielded strong agglutination reactions with all the strains which were suspected to possess the c_1 antigen, including strain 2253 The results were easily reproducible Absorption with mannitol salt agar cultures of strain 2253 exhausted the c_1 serum

It may accordingly be concluded that the c_1 factor serum should be prepared by absorption of serum 3647 with strain 2095 (and with 1503 if *n* antibodies are present), or by absorption of serum 1015 with strain 2095 Agglutination should be performed with mannitol salt agar cultures

Oeding's c serum (5) is obtained by further absorption of the *ac* serum with strain 1503, which removes a_1 and ac_1 antibodies The c serum has not been subjected to special analyses in these studies as it represented one of the reabsorbed *ac* sera

Distribution of the ac_1 and c_1 Antigens among the Type Strains

The agglutinability and absorbing capacity of the type strains, including two strains which will be described in the next article, have been presented in Table 5

TABLE 3

Agglutinability of Type Strains after Re-absorption of ac K 828 Serum

Agglutinating strain	*ac* serum K 828 absorbed with					*ac* serum unabsorbed
	365	3647	F 21	2095	1015	
365	—	—	—	—	+++	+++
3647	+++	—	+++	—	+++	+++
F 21	(+)	—	—	(+)	+++	+++
2095	+++	—	+++	—	+++	+++
Cowan III	+++	—	+++	+++	+++	+++
1015	+++	—	—	+++	—	+++
6376	+++	—	—	+++	—	+++

Code See Table 1

remove a_1 agglutinins A parellel absorption of the *a* serum was performed with the same batch of Wood 46 organisms to control the ab sorbing capacity of the strain Thereafter the absorbed *ac* serum was *re-absorbed* with the other strains Some agglutination patterns are presented in Table 2, which shows that strains F 21 and 830 absorbed ac_1 but not a_1 agglutinins Strains 1503, 2253, 28, 3647, 2095, Cowan III, and 6376 possessed both the a_1 and ac_1 *antigens*

Strong a_2, a_4, and a_5, and weak b_1 agglutinins were also demonstrated in the *ac* K 827 serum

No ac_1 antibodies were found in the ac serum K 828 In addition to strong a_4 and a_5 antibodies, this serum possessed strong b_1 antibodies, and antibodies to a new antigen represented by the strong agglutination of strains 1015 and 6376 The new antigen is designated c_1 After re-absorption of the *ac* serum with the type strains, five patterns of reactions were obtained as shown in Table 3 The absorption experiments showed that the following strains absorbed c_1 agglutinins 3647, F 21, *Cowan III, 1015, and 6376 The absorbing capacity of strains 3647* and F 21 varied considerably, howeever, as they in some experiments removed all antibodies while sometimes no absorption occurred

Preparation of Individual Factor Sera

Factor ac_1 serum The same difficulty was met with as before, that we have no strain for removal of a_4 agglutinins selectively from serum 3647 Moreover, we have no strain which absorbs *e*, *m*, and *n* antibodies from sera 1503, 2253, and 28 without removing possible ac_1 antibodies

Factor c_1 serum This serum can be prepared by absorption of serum 3647 (K 828) with strain 2095 If the resulting serum agglutinates strains 1503, 2253, and 28, this is most probably caused by the presence of *n* antibodies, and re-absorption should be performed with strain *1503 Strong c_1 factor* sera can also be obtained from sera 1015 and 6376 by absorption with strain 2095 (Table 4), but the 6376 serum contained in addition a weak specific antibody to the homologous strain

As mentioned above the absorbing capacity of strains 3647 and F 21

TABLE 4

Agglutination with Nutrient Agar and Mannitol Salt Agar Cultures in Three c_1 Sera

Strain	Ser 3647 abs with 2095		Ser 1015 abs with 2095		Ser 6376 abs with 2095	
	Nutrient agar	Mannit agar	Nutrient agar	Mannit agar	Nutrient agar	Mannit agar
53	—	+++	—	+++	—	+++
47*	—	+++	—	+++	—	+++
21*	(+)	+++	—	+++	(+)	+++
wan III	+++	+++	++	+++	+++	+++
15	+++	+++	+++	+++	+++	+++
76	+++	+++	+++	+++	+++	+++

Code * The strains were sometimes agglutinable also with nutrient agar cultures Cf otherwise Table 1

aried with regard to c_1 antibodies Also the agglutinability of these trains varied from one culture to another A weak agglutination with train 2253 was also occasionally observed The latter observation is of pecial interest, since strain 2253 is used for preparation of the *b* factor erum as the strain was previously found to absorb *c* antibodies (5) The c_1 antigen is heat-stable, but rather weak reactions were obtained with autoclaved bacteria Thus the c_1 antigen seems to be partially or ompletely blocked in some strains

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It may accordingly be concluded that the c_1 factor serum should be prepared by absorption of serum 3647 with strain 2095 (and with 1503 f *n* antibodies are present), or by absorption of serum 1015 with strain 2095 Agglutination should be performed with mannitol salt agar ultures

Oeding's *c* serum (5) is obtained by further absorption of the *ac* serum with strain 1503, which removes a_1 and ac_1 antibodies The *c* serum has not been subjected to special analyses in these studies as it represented one of the reabsorbed *ac* sera

Distribution of the ac_1 *and* c_1 *Antigens among the Type Strains*

The agglutinability and absorbing capacity of the type strains, including two strains which will be described in the next article, have been presented in Table 5

TABLE 5

Distribution of the ac_1 and c_1 Antigens among the Type Strains

Strain 1503	ac_1	
„ 2253	ac_1	c_1
„ 28	ac_1	
„ 3647	ac_1*	c_1
„ F 21	(ac_1)	c_1
„ 2095	ac_1*	
„ Cowan III	ac_1*	c_1
„ 830	(ac_1)	
„ 5687	(ac_1)	
„ 6376	(ac_1)	c_1
„ 1015		c_1

Code: ( ) The strain absorbs antibodies, but does not agglutinate
* The agglutinability of the strain is not known

Antigenic Formula of the Type Strains with Regard to a, b, ac, *and* c *Antigens*

The b_4 *and* b_5 antigens have not been included in the antigenic formulas given in Table 6 A bovine staphylococcus strain introduced by *Grun* (1958) for production of *l* antibodies, agglutinated strongly in the c_1 serum *Grun* (1) obtained, however, no agglutination in *c* factor serum

TABLE 6

Antigenic Formula of the Type Strains with regard to a b, ac, and c Antigens

Strain	Antigenic formula									
1503	a_1	b_2	ac_1							
2253	a_1	ac_1	c_1							
28	a_1	b_2	b_3	ac_1						
365	a_2	b_1	b_2							
3647	a_1	a_2	a_3	a_4	a_5	b_1	b_2	b_3	ac_1	c_1
F 21	b_1	b_2	ac_1	c_1						
17 A	a_2	a_5	b_3							
3189	a_3	b_2	b_3							
2095	a_1	a_2	a_3	a_4	a_5	b_1	b_2	b_3	ac_1	
Cowan I	a_2									
Cowan II	b_3									
Cowan III	a_1	a_2	a_3	a_4	a_5	b_1	b_2	ac_1	c_1	
670	a_2	a_3	b_3							
1015	c_1									
830	a_2	a_3	a_4	a_5	b_1	b_3	ac_1	c_1		
5687	a_1	a_2	a_3	a_4	a_5	b_1	b_2	b_3	ac_1	
6376	a_1	a_2	a_5	b_1	b_2	b_3	ac_1	c_1		
Wood 46	a_1									

Details regarding agglutinability and absorbing capacity of the strains have been given in connection with each factor serum

Comparison of the Antibody Compositions of the a, b, ac, *and* c *Factor Sera*

It has repeatedly been shown that the rabbit immune sera vary considerably with regard to antibody composition It is probable that most

TABLE 7

Antibody Composition of the a b, ac, and c Factor Sera

a serum	a_1	a_2	a_3	a_4	a_5							
b serum		a_2	a_3	a_4	a_5	b_1	b_2	b_3	b_4	b_5		
ac serum	a_1	a_2	a_3	a_4	a_5	b_1			b_4	b_5	ac_1	c_1
c serum		a_2		a_4	a_5	b_1				b_5		c_1

immune sera will lack one or more of the agglutinins The present list gives therefore only the antibody composition which theoretically has to be taken into account, when the factor sera are prepared The possibility that other immune sera may appear to contain new, hitherto unrecognized antibodies, should also be born in mind

DISCUSSION

The *ac* serum has been used in serological typing of staphylococci as a supplement to the *a* serum, which was found to be a rather weak serum (5) The same observation was made in the present investigations, but the absorption experiments showed that the stronger reactivity of strains 1503, 2253, and 28 in the *ac* serum was due to a specific antibody not present in the *a* serum The designation ac_1 was resorted to in accordance with the principles of the present registration, although the antigen has no connection to the *a* or *c* antigens The *ac* serum agglutinated most of the type strains (cf Table 1), and only 9 of 459 pyogenic staphylococci examined by *Oeding* (6) failed to agglutinate in the *ac* serum

Also the *c* factor serum possessed one specific agglutinin in addition to the agglutinins shared with *a* and *b* factor sera Strong agglutination reactions were obtained with the c_1 serum, especially when mannitol-salt agar cultures were used for agglutination An obviously pure c_1 serum can be prepared from serum 3647 or serum 1015 The c_1 antigen appears to be a "major" antigen, and the c_1 serum will probably prove to be useful in serological typing

The c_1 antigen of strain F 21 was sometimes blocked in ordinary nutrient agar cultures, and the strain showed varying absorbing capacity This may explain why strain F 21 sometimes absorbed agglutinins from the *a* serum in *Oeding*'s investigations (5) If so, his *a* serum must have contained some c_1 antibody

In the previous article it was reported that strain 2253 did not remove c_1 antibodies from serum 3647, and the *b* serum could therefore not be prepared from serum 3647 This was contrary to *Oeding*'s findings (5), but the discrepancy may easily be explained by the inconstant blocking of the strain as demonstrated in the present investigations

Recently *Cohen & Oeding* (1962) have compared the slide agglutination and the fluorescent antibody methods in serological typing of staphylococci The results with the *a* and *b* sera showed almost complete

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FROM THE BACTERIOLOGICAL DEPARTMENT OF SERAFIMERLASARETTET, STOCKHOLM (HEAD ARNE LITHANDER M.D.)

DETERMINATION OF THE CONCENTRATION OF PENICILLIN IN BLOOD PLASMA, CEREBROSPINAL FLUID AND BRAIN SUBSTANCE IN THE RABBIT

By

ARNE LITHANDER

Received 1 iii 63

Of importance in chemotherapy is the degree to which the prepara tion used is bound to the protein in the organism, particularly to blood plasma Investigations on this problem have been conducted in the case of antibiotics and a number of sulphonamides

They have revealed that the degree of binding not only varies from one preparation to another, but also in different animal species *Klein* (1) for example, reported that the activity of penicillin in human plasma was only 50 per cent of that in buffer solution

Studies have likewise been made on the binding of penicillin to cere brospinal fluid *Klein* stated that the effect of cerebrospinal fluid on penicillin was insignificant

When studying the binding of penicillin to plasma and to cerebro-spinal fluid, the various investigators used only one concentration of penicillin in a specific organic substance

Lithander & Lithander (2) investigated the passage of penicillin to cerebrospinal fluid and brain substance following intravenous injection in rabbits with experimentally produced staphylococcic meningitis They ... *Klein* to determ... In con nectio... s of the metho... this technique and on the manner in which penicillin is bound to plasma cerebrospinal fluid and brain substance in rabbits A report of this investigation is given in the present paper

MATERIAL AND METHODS

Plasma Blood was removed from the jugular ve...
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samples of cerebrospinal fluid were stored at —30 degrees C. Each one was checked for sterility before use. Samples from approximately 100 rabbits were then pooled.

Brain substance. The brain was removed under sterile conditions from five healthy rabbits which had recently bled to death. The cerebral ventricles were slit. Blood and cerebrospinal fluid were rinsed out with saline solution after which the brains were dried with wadding cellulose. Equal quantities of the five brains were pooled. The same weight of phosphate buffer solution (pH 6.5) was added to the pool. The mixture of brain substance and buffer solution was then ground together with sterile fine sand in a mortar. After thorough grinding and mixing of the brain substance and buffer solution, the sand and brain substance were separated by centrifuging. The supernatant fluid was used for the investigations.

Diffusion technique in agar was used for determination of the penicillin[1] content in plasma, cerebrospinal fluid and brain substance. A modification of the method described by Klein was applied using *subtilis* spores of the ATCC 6633 strain as test organism. The spores were suspended in melted agar (pH 6.5) in a concentration of 7,500 spores per ml of agar. The agar was then poured into Petri dishes with plane bottoms which were placed on a horizontal base. When the agar had solidified six cylindrical holes nine mm in diameter were punched in each plate. 0.06 ml of phosphate buffer solution (pH 6.5) of standard penicillin containing 0.12, 0.06 and 0.03 units per ml were introduced in three of these holes in each plate. The remaining three holes in each plate were then filled with 0.06 ml of buffer solutions of the penicillin to be tested or with solutions of penicillin in plasma, cerebrospinal fluid or brain substance.

To determine the reliability of the method, analyses of variance were made of the diameters of the inhibition zones secured for 0.03, 0.06 and 0.12 units respectively per ml of standard penicillin on 70 plates. If the concentration of the penicillin was held within these limits, the error of the method did not exceed 5 per cent.

The error of the method when this range of concentration is exceeded was determined by testing penicillin solutions containing 0.25 and 0.50 units per ml respectively in relation to the standard penicillin. In addition, penicillin solutions containing 0.03125, 0.0625 and 0.125 units per ml were compared with the standard penicillin.

Ten agar plates with 30 holes were used for each one of these five penicillin concentrations representing 30 determinations. In the investigation of penicillin dissolved in plasma, cerebrospinal fluid and brain substance, 15 determinations were made in the same manner for each penicillin concentration in each one of the different dilutions of plasma, cerebrospinal fluid and brain substance. For a more exact study of the effect of these different organic substances they were tested not only undiluted but also in several dilutions. Plasma was used both undiluted and in dilutions of 1/2.5, 1/5, 1/20, 1/40 and 1/80 and cerebrospinal fluid was used undiluted and in dilutions of 1/2, 1/4, 1/8 and 1/16. Brain substance could not be used undiluted but only in dilutions of 1/2, 1/4, 1/8 and 1/16. The determinations in all the dilutions of plasma were made with the penicillin concentrations 0.03125 to 0.50 units per ml and in the cerebrospinal fluid and brain substance with the penicillin concentrations 0.025 to 0.40.

In all these tests, readings were made following incubation at 37° C for 18 hours. Two diameters at right angles to the inhibition zone were measured in a magnifying apparatus. Only circular zones were approved. A linear regression line between penicillin concentration (log units) and diameters was calculated for the standard in each plate. With the aid of this line the penicillin concentrations corresponding to the diameters obtained in the investigations of the error of the method and in the investigations with plasma, cerebrospinal fluid and brain substance were calculated.

RESULTS

The results of the investigation on the error of the method are shown in Table 1.

There is a clear tendency for the observed values to be lower than the

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1 Benzyl penicillin (penicillin G) supplied by AB Kabi, Stockholm, was used through the investigation.

theoretical ones There is a gradual decrease of the accuracy of the assay in that the deviation increased from 0 3 to approximately 20 per cent This is the tendential error. The random error is given by the standard deviation of the 30 values for each dilution It varies from 4 to 11 per cent Both errors tend to rise with the penicillin concentration If necessary, a correction may be made for the tendential error There is a marked increase of the errors, particularly the tendential error, at the highest concentrations Consequently, no concentrations higher than 0 250 I U per ml are acceptable for practical use

TABLE 1

Errors of the Method Penicillin in Buffer Solution Thirty Determinations Statistically Significant Deviations of Observed Means from the Theoretical Values are Underlined

Theoretical values * Units of penicillin pr ml	Observed mean values Units of penicillin per ml	Deviation (tendential error)	Per cent deviation	Standard deviation (random error)	Per cent standard deviation
0 03125	0 03107	—0 00018	0 58	0 0014	4 5
0 0625	0 0623	—0 00020	0 32	0 0024	3 8
0 1250	0 1258	+0 0008	0 64	0 0063	5 0
0 250	0.236	—0 014	5 6	0 020	7 9
0 500	0 403	—0 097	19 4	0 056	11 3

* Th[illegible]

The results of the investigations with plasma, cerebrospinal fluid and brain substance are shown in Tables 2, 3 and 4

Tables 2, 3 and 4 reveal that the observed penicillin concentrations were almost invariably lower than anticipated This applied both to plasma, cerebrospinal fluid and brain substances and to practically all penicillin concentrations The deviations were *smallest* in the case of cerebrospinal fluid and brain substance. they amounted to at most 15 per cent in the highest concentration of these substance used, *i e* in undiluted cerebrospinal fluid and in a 50 per cent dilution of [illegible] substance In [illegible]

this [illegible]

In u[illegible] example, the deviation was greatest for the two smallest penicillin concentrations, 0 03125 and 0 0625 units per ml, respectively

However th[illegible]

tral [illegible]

0 12 [illegible] we calculate the quotient between the theoretical and the observed value in buffer solution for example

TABLE 2

Inhibition of the Penicillin Effect by Plasma Penicillin in Plasma and in Buffer Solution of Plasma Fifteen Determinations for Penicillin in Undiluted Plasma and in each Plasma Dilution Number of Cases: 15

Theoretical values Units of penicillin per ml	Plasma dilution	Observed mean values Units of penicillin per ml $\bar{x} \pm \sigma_{\bar{x}}$ σ_x	Tendential error = observed mean value theoretical value	Tendential error in per cent of theoretical value	Random error in per cent of theoretical value
	Undil	0 01567 ± 0 00027 0 00106	—0 01558	—49 8	3 39
	1/2 5	0 02070 ± 0 00071 0 00273	—0 01055	—33 8	8 73
	1/5	0 02310 ± 0 00080 0 00308	—0 00815	—26 08	9 86
0 03125	1/10	0 02580 ± 0 00091 0 00353	—0 00545	—17 44	11 30
	1/20	*0 02660 ± 0 00066* 0 00256	*—0 00465*	*—14 88*	*8 19*
	1/40	0 0326 ± 0 0006 0 0024	+0 00135	+ 4 32	8 00
	1/80	0 0301 ± 0 0004 0 0016	—0 00115	— 3 68	5 12
	Undil	0 03733 ± 0 00050 0 00194	—0 02517	—40 3	3 10
	1/2 5	0 05160 ± 0 00106 0 00409	—0 01090	—17 44	6 54
	1/5	0 05220 ± 0 00126 0 00488	—0 01030	—16 48	7 81
0 0625	*1/10*	*0 05493 ± 0 00137* 0 00530	*—0 00757*	*—12 11*	*8 48*
	1/20	0 05520 ± 0 00169 0 00423	—0 00730	—11 68	6 77
	1/40	0 0600 ± 0 0010 0 0039	—0 0025	— 4 00	6 24
	1/80	0 0589 ± 0 0007 0 0028	—0 0027	—4 32	4 48
	Undil	0 08987 ± 0 00240 0 00928	—0 03513	—28 10	7 42
	1/2 5	0 10527 ± 0 00328 0 01270	—0 01973	—15 78	10 16
	1/5	0 11247 ± 0 00382 0 01480	—0 01253	—10 02	11 84
0 125	1/10	0 11600 ± 0 00297 0 01150	—0 00900	— 7 20	9 20
	1/20	0 11400 + 0 00307 0 01190	—0 01100	— 8 80	9 52
	1/40	0 1210 ± 0 0020 *0 0076*	—0 0040	3 20	6 08
	1/80	0 1221 ± 0 0015 0 0057	—0 0029	— 2 32	4 56

TABLE 2 (cont.)

Theoretical values Units of penicillin per ml	Plasma dilution	Observed mean values Units of penicillin per ml $\bar{x} \pm \sigma_{\bar{x}}$ σ_x	Tendential error = observed mean value − theoretical value	Tendential error in per cent of theoretical value	Random error in per cent of theoretical value
	Undil	0.18233 ± 0.00555 0.02150	−0.06767	−27.67	8.60
	1/2.5	0.21600 ± 0.00457 0.01770	−0.03400	−13.60	7.08
	1/5	0.21533 ± 0.00517 0.0200	−0.03467	−13.87	8.00
0.25	1/10	0.22667 ± 0.00548 0.02120	−0.02333	− 9.33	8.48
	1/20	0.23400 ± 0.00517 0.02000	−0.01600	− 6.40	8.00
	1/40	0.2460 ± 0.0037 0.0142	−0.0040	− 1.60	5.68
	1/80	0.2373 ± 0.0032 0.0123	−0.0127	− 5.08	4.92
	Undil	0.33800 ± 0.01168 0.04520	−0.16200	−32.40	8.50
	1/2.5	0.37133 ± 0.01692 0.06550	−0.12867	−25.73	13.10
	1/5	0.38400 ± 0.01599 0.06190	−0.11600	−23.20	12.38
0.5	1/10	0.40600 ± 0.01411 0.05460	−0.09400	−18.80	10.92
	1/20	0.41267 ± 0.01320 0.05110	−0.08733	−17.47	10.22
	1/40	0.3907 ± 0.0159 0.0615	−0.1093	−21.86	12.30
	1/80	0.4033 ± 0.0089 0.0343	−0.0967	−19.34	6.86

with a dose of 0.250 the quotient would be 0.250 0.236 = 1.059, the figure secured can be used in working out a corrected analytical value, which in turn can be related to the theoretical value with different doses and different dilutions in a body fluid Table 5 and Figure 1 illustrate the last mentioned ratio for different concentrations of penicillin in different dilutions of plasma The ratio is labelled yield and is given in per cent

It appears from Table 5 that the yield rose with an increase in the theoretical concentration of *penicillin* This circumstance was most pronounced when undiluted plasma was used

When penicillin was dissolved in plasma, it was found that even with a dilution of 1:2.5 the yield was almost total when the theoretical penicillin concentration was 0.5 and 0.25 units per ml, respectively With lower theoretical penicillin concentrations *i e* 0.03125 to 0.125,

TABLE 3

Inhibition of the Penicillin Effect by Cerebrospinal Fluid Penicillin in Cerebrospinal Fluid and in Buffer Solution of Cerebrospinal Fluid Fifteen Determinations for Penicillin in Undiluted Cerebrospinal Fluid and in each Dilution thereof Number of Cases 15

Theoretical values Units of penicillin per ml	Cerebrospinal fluid dilution	Observed mean values Units of penicillin per ml $x \pm \sigma_x$ σ_x	Tendential error = observed mean value theoretical value	Tendential error in per cent of theoretical value	*Random* error in *per cent* of theoretical value
	Undil	0 02187 ± 0 00043 0 00165	—0 00313	—12 52	6 60
	1/2	0 02233 ± 0 00060 0 00205	—0 00267	—10 68	8 20
0 025	1/4	0 02333 ± 0 00044 0 00171	—0 00167	— 6 68	6 84
	1/8	0 02253 ± 0 00049 0 00189	—0 00247	— 9 88	7 56
	1/16	0 0246 ± 0 0003 0 0011	—0 0004	— 1 60	4 40
	Undil	0 04487 ± 0 00110 0 00424	—0 00513	—10 26	8 48
	1/2	0 04740 ± 0 00144 0 00558	—0 00260	— 5 20	11 16
0 05	1/4	0 04887 ± 0 00082 0 00319	—0 00113	— 2 26	6 38
	1/8	0 04827 ± 0 00078 0 00304	—0 00173	— 3 46	6 08
	1/16	0 0491 ± 0 0008 0 0032	—0 0009	— 1 80	6 40
	Undil	0 08587 ± 0 00251 0 00972	—0 01413	—14 13	9 72
	1/2	0 08627 ± 0 00196 0 00758	—0 01373	—13 73	7 58
0 10	1/4	0 08920 ± 0 00166 0 00644	—0 01080	—10 80	6 44
	1/8	0 09467 ± 0 00171 0 00663	—0 00533	— 5 33	6 63
	1/16	0 1029 ± 0 0022 0 0085	+0 0029	+ 2 90	8 50
	Undil	0 18933 ± 0 00561 0 02170	—0 01067	— 5 34	10 85
	1/2	0 18900 ± 0 00434 0 01680	—0 01100	— 5 50	8 40
0 20	1/4	0 19933 + 0 00331 0 01280	— 0 00067	— 0 33	6 40
	1/8	0 1953 ± 0 0046 0 0177	—0 0047	— 2 35	8 85
	1/16	0 1930 ± 0 0028 0 0108	—0 0070	3 50	5 40
	Undil	0 37200 ± 0 00602 0 02330	—0 02800	— 7 00	5 83
	1/2	0 36867 ± 0 00749 0 02900	—0 03133	— 7 83	7 25
0 40	1/4	0 37133 ± 0 00703 0 02720	—0 02867	— 7 17	6 80

TABLE 4

Inhibition of the Penicillin Effect by Brain Substance. Penicillin in Brain Substance and in Buffer Solution thereof Fifteen Determinations for Penicillin in Undiluted Brain Substance and in each brain Substance Dilution Number of Cases: 15

Theoretical values Units of penicillin per ml	Brain substance dilution	Observed mean values Units of penicillin per ml $\bar{x} \pm \sigma_{\bar{x}}$ σ_x	Tendential error = observed mean value-theoretical value	Tendential error in per cent of theoretical value	Random error in per cent of theoretical value
0 025	1/2	0 02153 ± 0 00053 0 00207	−0 00347	−13 88	8.28
	1/4	0 02207 ± 0 00062 0 00240	−0 00293	−11 72	9 60
	1/8	0 02287 ± 0 00065 0 00253	−0 00213	− 8 52	10 12
	1/16	0 0251 ± 0 0003 0 0012	+0 0001	+ 0 40	4 80
0 05	1/2	0 04240 ± 0 00135 0 00521	−0 00760	−15 20	10 42
	1/4	0 04360 ± 0 00076 0 00295	−0 00640	−12 80	5 90
	1/8	0 04527 ± 0 00057 0 00222	−0 00473	− 9 46	4 44
	1/16	0 0499 ± 0 0069 0 0268	−0 0001	− 0 20	5 36
0 10	1/2	0 09320 ± 0 00189 0 00730	−0 00680	− 6 80	7 30
	1/4	0 09173 ± 0 00141 0 00546	−0 00827	− 8 27	5 46
	1/8	0 10027 ± 0 00122 0 00473	+0 00027	+ 0 27	4 73
	1/16	0 0997 ± 0 0019 0 0074	−0 0003	− 0 30	7 40
0 20	1/2	0 18333 ± 0 00398 0 01540	−0 01667	− 8.33	7 70
	1 4	0 18267 ± 0 00548 0 02120	−0 01733	− 8 66	10 60
	1,8	0 18033 ± 0 00439 0 01700	−0 01967	− 9 83	8 50
	1/16	0 1987 ± 0 0024 0 0093	−0 0013	− 0 65	4 65
0 40	1/2	0 34200 ± 0 01132 0 04380	−0 05800	−14 50	10 95
	1/4	0 33867 ± 0 01062 0 04110	−0 06133	−15 33	10 27
	1/8	0 34333 ± 0 00951 0 03680	−0 05667	−14 17	9 20
	1/16	0 3760 ± 0 0093 0 0358	−0 0240	− 6 00	8 95

TABLE 5

The Yield when Penicillin is Dissolved in Rabbit Plasma The Theoretical Concentration of Penicillin was Varied in the Different Plasma Dilutions between 0 03125 and 0 50 I U per ml The Plasma Dilutions Ranged between 1/1 and 1 80 Yield in per Cent

Plasma dilution	Pt(*) = 0 03125 Q(†) = 1 006	Pt = 0 0625 Q = 1 003	Pt = 1 1250 Q = 0 991	Pt = 0 2500 Q = 1 059	Pt = 0 5000 Q = 1.21
Undil	50 4	59 9	67 9	77 3	83 8
1/2 5	66 6	82 8	79 5	91 6	92 1
1/5	74 4	83 8	84 9	91 3	95 2
1/10	83 1	88 2	87 6	96 1	100 7
1/20	85 6	88 6	86 1	99 2	102 3
1/40	104 9	96 3	91 4	104 3	96 9
1/80	96 9	94 5	92 2	100 6	100 0

* Pt = theoretical concentration of penicillin Units per ml

† Q = the ratio between the theoretical and the observed penicillin concentration in buffer solution

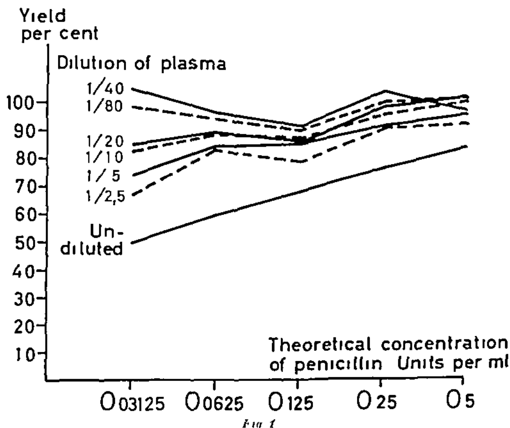


Fig 1

Yield when penicillin in different concentrations is dissolved in rabbit plasma with different degrees of dilution

a similar yield was first found in a plasma dilution of 1/40 The yield in undiluted plasma was much smaller for these lower penicillin concentrations An increase in the yield was observed when the dilution of plasma was *increased*

A corresponding direct calculation cannot be made for cerebrospinal fluid and brain substance, since the investigation on the error of the method was carried out with a different penicillin concentration than the ones used in the experiments with cerebrospinal fluid and brain. However, the true yield in cerebrospinal fluid and brain substance was estimated by interpolation in the values for yield when the penicillin concentration was determined in buffer solution. For example the yield appears to increase in these substances also with increasing penicillin concentration and with increasing dilution of cerebrospinal fluid and brain substance.

In the case of cerebrospinal fluid, the yield appeared to be reduced only with 0.025, 0.05 and 0.10 units of penicillin per ml in undiluted cerebrospinal fluid and with 0.025 units also in fluid diluted 1/2. In brain, a comparable reduction of yield probably only occurs with penicillin concentrations of 0.025 and 0.05 units per m, respectively, in dilutions of 1/2 and 1/4 of brain substance.

DISCUSSION

In the determination of the penicillin content of the blood plasma following the administration of penicillin in man or animals, only the penicillin unbound to plasma can be evaluated by biological methods.

It has been shown that the capacity to bind penicillin to blood plasma differs at least in some animal species. In certain situations, it is probably only the unbound penicillin which is of *significance, e.g.* in infections of the meninges while under other circumstances the total concentration in plasma may be of interest.

It has been shown in the present investigation that plasma, cerebrospinal fluid and brain substance in the rabbit have the capacity to *bind penicillin*. This capacity appears to be expressed in varying degree, depending on the concentration aimed at in the different substances of the organism following administration of penicillin.

By determining the concentration of unbound penicillin in plasma, it should be possible in rabbits to evaluate within some degree of accuracy the total concentration of penicillin from the estimated penicillin concentration in the buffer solution.

As anticipated, the binding of penicillin to cerebrospinal fluid and brain substance was less than to plasma.

SUMMARY

1. A modification of *Klein's* diffusion method for the determination of penicillin is described. The technique permits the use of as small samples as 0.06 ml.

2. The practicability of this method for the determination of the

penicillin concentration in plasma, cerebrospinal fluid and brain substance in the rabbit was tested

3 The method was used to determine the penicillin not bound to plasma, cerebrospinal fluid and brain substance, but at the same time it permits an evaluation of the total concentration of penicillin in these substances

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FROM THE DEPARTMENT OF NEUROLOGY, AARHUS KOMMUNEHOSPITAL AND
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DENMARK

ANTIBODY IN MAN AGAINST A BOVINE STRAIN OF PARA-INFLUENZA VIRUS TYPE 3

By

CARSTEN RINDOM SCHIØTT

Received 5 iii 63

We have previously reported on the isolation of a bovine strain of para-influenza type 3 from a cow with an illness of the mucosal disease complex (9, 10) The virus can be grown in tissue cultures of calf kidney and guinea pig kidney, and it is pathogenic for suckling mice after intracerebral inoculation Sera from cattle reveal antibodies in a large proportion of cases (11), and as will appear from this report sera from human beings also contain antibody

MATERIALS AND METHODS

Virus Mater

Neutraliz

virus varyi ... to each dilution was added an equal volume of virus dilution containing approx 300 TCD_{50} per 0 1 ml The serum virus mixtures were incubated at 37° C for 30 minutes and then inoculated in 0 1 ml amounts into each of three tissue culture tubes Readings were made when the virus control gave a full CPE The antibody titre was considered to be 1 8 (1 32 or 1 128) if two out of the three tissue cultures were neutralized When only one culture tube showed neutralization the antibody titre was taken as less than 1 8 (1 32 or 1 128) and higher than the serum dilution when all three tubes were neutralized

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penicillin concentration in plasma, cerebrospinal fluid and brain substance in the rabbit was tested

3 The method was used to determine the penicillin not bound to plasma, cerebrospinal fluid and brain substance, but at the same time it permits an evaluation of the total concentration of penicillin in these substances

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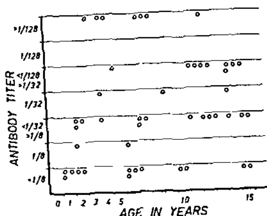


Fig 2
PI 3 antibody titre of 48 children's sera

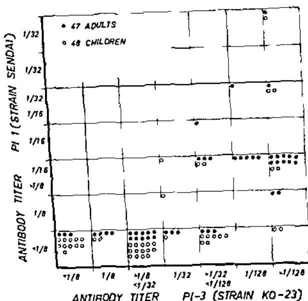


Fig 3
CPE neutralizing titres against PI 1 (Sendai) and PI 3 (KO 23) viruses

to be some correlation between the neutralization of the two viruses 17 per cent of the sera were able to neutralize PI 3 virus in dilutions 1 32 or higher without having any effect on the PI-1 strain

In Fig 4 the neutralizing antibody titres are compared with the haemagglutination inhibition titres of 43 sera from adults Correlation between neutralizing and HI antibodies in individual sera was present in the sense that subjects possessing high titres of neutralizing antibody also had high titres of HI antibody

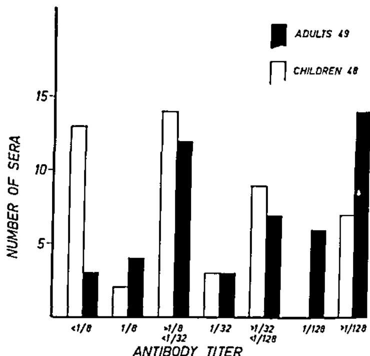


Fig 1

Sera distributed according to PI-3 antibody level

dilutions of serum in saline were added equal amounts (0 25 ml) of PI 3 virus diluted to contain 4 HA units The serum-virus mixtures were then incubated at 37° C for 30 minutes After addition of 0 5 ml of a 0 25 per cent suspension of cow erythrocytes the titration series were kept at 4° C overnight The HI titre was taken as the highest dilution of serum which completely inhibited agglutination

Sera Two groups of sera were examined 49 sera from adults (18 56 years old) and 48 sera from children (1–15 years old) The adults were healthy donors chosen at random, and the children's sera were obtained from the Central Laboratory of Aarhus Kommunehospital All the children were patients in the hospital, the majority in the Department of Neurology and a few in the E N T Department None of the children were known to suffer from acute respiratory tract infections The sera were heated to 56° C for 30 minutes and then stored at —20° C

RESULTS

In Fig 1, the 97 sera are distributed according to the antibody level Of the children, 13 (27 per cent) had no antibody (< 1 8) as compared with only three of the adults (6 per cent) About 40 per cent of the sera from adults were able to neutralize PI 3 virus in dilutions 1 · 128 or higher, whereas only 15 per cent of the children's sera had the same capacity Sera with antibody titres 1 8 or higher were found in 84 per cent of the 97 persons studied

In Fig 2, the antibody titres are related to the ages of the children studied. It is seen that high titres are absent only in the age group up to 2 years.

The neutralizing capacity of 95 sera against PI 3 virus was compared with the neutralization of PI–1 virus (Fig 3) Although there seems

TABLE 1

Distribution of Neutralizing Antibody for PI 1 and PI 3 Viruses in Different Populations. A Comparison between our Result and that of La Placa & Moscovici (8)

Population	PI 1 (Sendai)			PI-3 (HA 1)			PI-3 (KO 23)		
	No sera tested	With antibody ≥ 1:8*)		No sera tested	With antibody ≥ 1:8*		No sera tested	With antibody ≥ 1:8	
		No	%		No	%		No	%
American adults	50	38	76.0	50	50	100.0			
American children	18	8	44.4	43	30	69.7			
American total	68	46	67.6	93	80	86.0			
Italian adults	59	48	81.5	70	68	97.0			
Italian children	61	32	52.4	70	65	92.0			
Italian total	120	80	66.7	140	133	94.9			
Indian adults	45	31	68.8	40	39	97.5			
Danish adults	47	24	51.1†				49	46	93.9†
Danish children	48	10	20.8				48	35	72.9
Danish total	95	34	35.8				97	81	83.5

* Haemadsorption inhibition test
† Neutralization of CPE

According to the above considerations, it should be possible to test human sera for PI-3 antibody using a bovine strain as antigen. Only a few sera would not turn out to be positive, *viz.* those from persons having had only one infection with human PI 3 strains.

La Placa & Moscovici (8) studied the distribution of para influenza antibody in different groups of populations; their results are compared with ours in Table 1. The distribution of PI 3 antibody in a Danish group corresponds to that obtained by *La Placa & Moscovici* and *Deibel et al.* (4). The percentage of PI-1 positive sera in the Danish group is lower than in the other groups mentioned and it is also lower than the values reported by *Versteeg* (12). In a Dutch population he found 1449 HI positive sera among 2149, *i.e.* 70 per cent positive sera. Our result with PI 1 virus is more in accordance with that obtained by *Hilleman* (6) who found that only 25 per cent of children had detectable neutralizing antibody against PI 1 virus.

PI 1 and PI 3 viruses are distinct from each other, but nevertheless natural infection (*Chanock et al.* (3)) and vaccination (*Jensen et al.* (7)) with one or the other *type will frequently cause a heterologous* antibody rise. Therefore it is not possible to estimate the frequency of PI 1 and PI 3 infections merely on the basis of a serological examination of sera. Of 48 children (Fig. 3), 10, or 21 per cent had antibody titres 1:32 or higher against PI-3 virus and less than 1:8 for PI 3 virus. The corresponding figure for adults was 6 or 13 per cent. It is interesting to note that in no case was a high antibody titre against PI 1 virus associated with a low PI-3 antibody level.

HI

ANTIBODY TITER

1/64 1/32 1/16 1/8 <1/8

<1/8 1/8 >1/8 <1/32 1/32 >1/32 <1/128 1/128 >1/128

ANTIBODY TITER NEUTRALIZATION

Fig 4

HI antibody and PI neutralizing antibody titres of 43 sera from healthy donors

DISCUSSION

The antigenic relationship of the para-influenza viruses seem very complicated *Abinanti & Huebner* (1) found that human and bovine strains of the PI–3 group were antigenically indistinguishable Later *Hamparian & Hilleman* (5) showed that the viruses could be differentiated by the use of serum from intranasally infected guinea pigs, but they also observed that the specific response to infection was lost after repeated exposure to the same virus These findings were subsequently confirmed by *Abinanti et al* (2) in experiments with guinea pigs They also found that the antibody responses of four of five children undergoing the first natural infection with a human strain of PI–3 virus were similar to those of the guinea pigs, *i e* a specific response to the homologous (human) strain The fifth child, however, developed the same level of antibody to the human and bovine strain of the PI–3 virus After a second natural infection in the same four children with PI–3 virus, one child developed a low level of antibody to the bovine strain, and in two other children bovine antibody was present in the same titre as human strain antibody

K E Jensen et al (7) investigated the antigenicity of PI 1 and PI 3 strains After intranasal infection of ferrets, the convalescent sera were studied for the presence of homologous and heterologous antibody by the haemagglutination-inhibition test There were no cross-reactions within the two PI 1 strains, whereas the two PI 3 strains a bovine and a human strain—were closely related The bovine PI 3 strain was antigenetically broader than the human strain, *i e* that the heterologous antibody titre was higher after an infection with the bovine strain than with the human strain, although never as high as the homologous antibody titre There were no cross reactions between PI 1 and PI 3 strains

TABLE 1

Distribution of Neutralizing Antibody for PI 1 and PI 3 Viruses in Different Populations A Comparison between our Result and that of La Placa & Moscovici (8)

Population	PI 1 (Sendai)			PI 3 (HA 1)			PI 3 (KO-23)		
	No sera tested	With antibody ≥ 1 8*)		No sera tested	With antibody ≥ 1 8*		No sera tested	With antibody ≥ 1 8	
		No	%		No	%		No	%
American adults	50	38	76 0	50	50	100 0			
American children	18	8	44 4	43	30	69 7			
American total	68	46	67 6	93	80	86 0			
Italian adults	59	48	81 5	70	68	97 0			
Italian children	61	32	52 4	70	65	92 0			
Italian total	120	80	66 7	140	133	94 9			
Indian adults	45	31	68 8	40	39	97 5			
Danish adults									
Danish children									
Danish total							[illegible]	[illegible]	[illegible]

* Haemadsorption inhibition test
† Neutralization of CPE

According to the above considerations, it should be possible to test human sera for PI-3 antibody using a bovine strain as antigen Only a few sera would not turn out to be positive *viz* those from persons having had only one infection with human PI-3 strains

La Placa & Moscovici (8) studied the distribution of para influenza antibody in different groups of populations their results are compared with ours in Table 1 The distribution of PI 3 antibody in a Danish group corresponds to that obtained by *La Place & Moscovici* and *Deibel et al* (4) The percentage of PI-1 positive sera in the Danish group is lower than in the other [illegible] values reported by *Vers* [illegible]

[illegible] *Hilleman* [illegible] had detectable neutralizing antibody against PI 1 virus

PI 1 and PI-3 viruses are distinct from each other, but nevertheless natural infection (*Chanock et al* (3)) and vaccination (*Jensen et al* (7)) with one or the other type will frequently cause a heterologous antibody [illegible] timate the frequency of PI 1 a[illegible] a serological examination of [illegible] per cent, had antibody titres 1 32 or higher against PI-3 virus and less than 1 8 for PI 3 virus The corresponding figure for adults was 6 or 13 per cent It is interesting to note that in no case was a high antibody titre against PI-1 virus associated with a low PI-3 antibody level

SUMMARY

A bovine strain (KO–23) of PI–3 virus was used as antigen in neutralization tests with human sera Antibody was present in a large proportion of cases, i e that 94 per cent of the adults and 73 per cent of the children had titres of 1 8 or higher Of the children, 15 per cent had titres 1 128 or higher as compared to 40 per cent of the adults Absence of high titres was found only among children aged from 1 to 2 years, thus indicating an early infection Antibody against PI–1 virus (Sendai) was less frequent, with a total of 36 per cent positive sera as against 84 per cent for PI–3 virus

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UPPSALA SWEDEN

HUMAN COMPLEMENT COMPONENTS IN STARCH-GEL ELECTROPHORESIS

2 *Experiments in Isolation and Identification by Preparative Electrophoresis*

By

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In a previous work experiments have been described, which provide some indication that two serum fractions evident in starch-gel electrophoresis are correlated to the complement system (1) The fractions are found in the so-called alpha beta globulin region, migrate slower than transferrin and can be demonstrated in all sera independently of their haptoglobin group There seemed to be good reason for supposing that the more rapidly migrating fraction might contain C'1 and the slower fraction C 2

In Hp 1 1 and 2 1 sera the fastest haptoglobin moves together with the more rapidly migrating fraction and in Hp 2–2 sera both fractions are free of haptoglobin In umbilical cord sera from the newborn, w[illegible] tv[illegible] F[illegible] evident that the more rapidly migrating fraction in Hp 1 1 and 2–1 sera consisted of at least two sub components, of which one was haptoglobin The question whether the more slowly migrating fraction is also inhomogeneous must be left open Because the functions of the fractions are unknown and because they are, at least to the eye homogeneous, the more slowly migrating fraction has been called X1 and the one moving more rapidly X2, (see Fig 4)

The electrophoretic method of separating plasma proteins is of the utmost importance in protein chemistry The method has also given valuable information concerning the quantitative and qualitative pattern of the plasma proteins in health and disease and in this way has contributed to the diagnosis of a number of different diseases New electrophoretic techniques with an increased separation capacity, for example electrophoresis in gels, are likely to afford even greater possibilities in these respects But at the same time it is necessary to increase our knowledge of the functions of these newly discovered plasma-

protein fractions. The functions of X1 and X2, studies of which are reported in this paper, are unknown, except that X2 may contain haptoglobin as one component.

There is a great deal of literature on the binding of complement to immune aggregates, and in particular to sensitized erythrocytes. The sequence of changes in such interactions and the kinetic aspects of immune haemolysis (3, 4) have likewise attracted much attention. A large number of observations on the changes of complement status in different diseases has also been published (reviewed in 3 and 4). The protein nature of the complement factors seems to be generally accepted (3) but because they occur in such small amounts in serum and also because they are so labile towards the preparative treatment which, for instance, isolation experiments imply, they have as yet not been isolated in a form sufficiently pure for physico-chemical description (5, 6). The glycoprotein β_{1C}, however, is probably identical with one of the C3 components (7). Thus at present complement factors can only be studied by indirect methods. They are, with the exception of the case stated above, still unknown serum factors and can not be localized to definite fractions in electrophoresis with high resolution, such as, for example, starch gel electrophoresis.

The purpose of the present communication is to report work on attempts to correlate one or more of the complement factors either to X1, X2 or to other zones in the electropherogram.

MATERIAL AND METHODS

Serum. Individual samples or pooled samples of fresh serum from healthy human blood donors were obtained and used immediately in the experiments.

For preparative starch gel electrophoresis the serum was concentrated by the freeze thawing method (8) 3–4 times by which method the complement titres were also increased correspondingly as compared with those in unconcentrated serum. Because of the poor yield of complement in the eluted serum fractions from the starch gel it was desirable to begin with as high an initial complement titre as possible.

R2 reagent, i.e. euglobulin and R1 reagent, i.e. pseudoglobulin were prepared from fresh serum by gel filtration (9) and used in preparative starch gel electrophoresis. R2 was also used as a starting material for the preparation of β_{1C}.

Preparation of β_{1C} and 11S Protein

β_{1C} was prepared according to *Muller Eberhard et al.* (10) by DEAE cellulose anion exchange chromatography of the serum euglobulin fraction.

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The role of β_{1C} in immune haemolysis has previously been described (6). 11S globulin has been shown to be an essential complement component in immune haemolysis (11). It is bound to the sensitized erythrocytes (EA complex) before C'1 and the reaction does not require the presence of cations. It has also been shown that the complement reagent R1, which is used to determine C'1, lacks the 11S component.

In order to determine isolated C'1 with R1 in the experiments described below it was therefore necessary to introduce 11S into the reaction. It should also be

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[illegible] fraction

Elution of Protein from Starch Gel

[illegible]

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Preparative starch gel electrophoresis was carried out in accordance with the principles established by *Smithies* (12) The type of starch the size of gel container the electrophoresis time and temperature were the same as given in a previous publication (1) A number of different buffer systems were tested and the final conditions used are given together with the results

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bound to the gel For this reason some preliminary investigations on the effect of the freezing temperature and duration of freezing on protein recovery were made

TABLE 1

Preparative Starch Gel Electrophoresis Subsequently the Gel Was Frozen and Thawed and the Protein Removed by Centrifugation The Protein Recovery Was Related to the Freezing Temperature and Freezing Time

Time	Per cent recovery		
	—18 C	— 70 C	—180 C
10 sec			13
30 „			20
60 „			15
3 min			13
10 „			11
30 „			9
0 „		12	
15 „		46	
30 „		56	
1 hour	52	58	
2 „		51	
4 „	46	51	
6 „	35		
8 „	37		
22	35	60	

When electrophoresis was completed, the gel was cut parallel with the direction of movement into pieces of equal size Each piece thus contained the whole of the separated region from gamma globulin to prealbumin The application region was excluded from the investigation Table 1 gives the results, which shows that a freezing temperature of —18° C or —70° C gave a recovery of 50 60 per cent, while freezing to —180° C (liquid nitrogen) yielded only a third of this value The maximum recovery at temperatures of —18° C and —70° C was obtained after about one hour's freezing, and did not increase with longer freezing times At —18° C there was a reduction of the recovered amount if the freezing time exceeded 4 hours From the results of these experiments it was decided to freeze the gel to —70° C for 15–30 minutes

B *Factors Influencing the Complement Recovery*

Whole serum introduced into starch gel prepared with the tris-citrate acid buffer of *Poulik* (16) and subsequently stored at +4° C for 24 hours lost about 50 per cent of its complement When the serum was separated electrophoretically in the gel, complement activity of the eluted serum fractions was at first either insignificant or absent It was therefore necessary to determine which factors were required to safeguard complement activity during electrophoretic separation

It is known that several factors, such as pH, ionic strength and temperature (17), ascorbic acid (18) and sorbitol (19) affect the stability of complement *in vitro*. A group of substances are used as protein stabilizers and some of them may possibly also protect complement. To this category belong glycine, mercaptoethanol, *p*-mercuric benzoate and Triton X–100.

The possible significance of all these factors for complement recovery from starch gel has been studied. As complement is best preserved at pH 6.4–7.0 (17), some electrophoretic separations were made with phosphate buffer in this pH range. As expected, a poor separation was obtained after very prolonged electrophoresis time and complement was not demonstrable in those fractions which had migrated out. An experiment in which the ionic strength of the tris-citrate acid buffer, with which the gel was cast, was doubled required a lower current to avoid excess heating in the gel, and thereby required excessive electrophoresis time. In such a gel an increased complement recovery was not obtained. The presence of sorbitol, glycine, mercaptoethanol, Triton X 100 or *p*-mercuric benzoate in the gel and electrolytic vessel did not increase complement recovery. It is possible, however, that ascorbic acid had some protecting effect. Since starch is a large molecular polysaccharide and it is known that such substances have a C'3-inactivating effect, possibly via a properdin polysaccharide complex in the presence of cations, it seems quite possible that during its migration in the gel complement becomes inactivated by such a mechanism. The addition of the cation binding EDTA to the gel and electrolyte vessel should thus hinder the fixation of complement to a starch properdin complex and in this way increase complement recovery. Whether or not this explanation is right, it was found that addition of EDTA to the gel and electrolyte vessel increased the recovery of the complement components C'1, C'2 and C'4. In order, however, that the separation pattern obtained with tris citrate acid buffer alone should not be altered, the EDTA concentration must be kept as low as possible. It was found that 0.0005 M EDTA in the gel and electrolyte vessel was sufficient to give increased recovery. Fig. 1 shows an experiment in which the C'2 and C'4 recoveries from the gels prepared differently were investigated. Gel 1 was prepared with tris citrate acid buffer containing 0.0005 M Na_2 EDTA and 20 mg ascorbic acid/100 ml buffer. Gel 2 was prepared with tris citrate acid buffer alone. Each gel was divided into three equal pieces, which were frozen for different periods. From the figure it can be seen that Gel 1 clearly gave a higher recovery than Gel 2 for both C'2 and C'4, but that the different freezing times did not change the results. A similar experiment using ascorbic acid alone in one of the gels resulted in no obvious difference in complement recovery.

The electrophoresis time was of great importance. If longer than 5 hours, complement activity was in general absent in the eluted fractions. Experiments with continuous gradient buffer systems and long electro

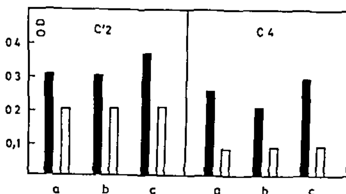


Fig 1

Preparative starch gel electrophoresis Recovery of complement factors C 2 and C 4 Solid bars electrophoresis in the presence of 0 0005 M EDTA Unfilled bars without EDTA Freezing time before thawing (*a*) 10 minutes (*b*) 30 minutes and (*c*) 120 minutes Ordinate optical density of the haemolysates at 541 mμ

phoresis time in attempts to separate the electrophoretic fractions more thoroughly always failed as regards the presence of complement activity

In an attempt to demonstrate complement in eluted fractions from an electrophoretically separated R2 (serum euglobulin fraction) it was found, however, that the presence of EDTA did not increase complement recovery Finally it was established that the eluates had a mild anti-complementary action, thus masking the real complement contents

The investigations concerning factors which might be important in the recovery of complement from starch-gel-separated serum and euglobulin thus yielded the finding that (*a*) the electrophoresis time should not exceed 5 hours, (*b*) the presence of EDTA increases recovery and (*c*) neither known complement stabilizing nor general protein-protecting factors were shown to have any importance

C *The Localization of Complement Factors to Electrophoretic Zones*

A number of experiments with the elution of protein from different parts of the electrophoretic separation region (including the gamma globulin and the prealbumin) showed that the complement activity was only found in the region from β_{1C} to transferrin The complement factors demonstrated were C 1, C 2, C 4 and β_{1C} (measured with guinea-pig R3) With human R3 reagent no C 3 activity was found, not even if all the eluted fractions were combined in all conceivable ways

Attention was therefore concentrated on this complement bearing region Fig 2 illustrates an experiment in which after electrophoresis this region was divided into six parts of equal size (about 1 5 mm broad gel strips) These strips were first frozen and thawed and protein was then eluted from them, the complement activity in these eluates being measured with complement reagents R1, R2 and R4 The different complement activities are given in the form of bars alongside the schematic electropherogram It can be seen in the figure that the

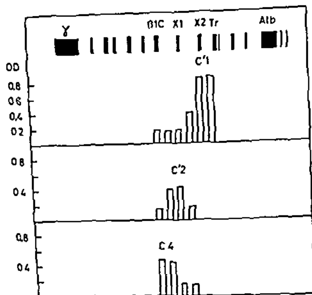


Fig 2

Preparative starch gel electrophoresis of serum After electrophoresis the block was divided between the β_{1C} and transferrin regions into pieces of equal size which were then frozen thawed and eluted by centrifugation The complement activity of the eluates was then determined A schematic diagram of the electrophoretic picture is given in the upper part of the diagram The lower part shows the activity of C 1 C 2 and C 4 in each eluate Ordinate optical density of the haemolysate at 541 mμ

maximum for C 2 activity corresponds very well with the electrophoretic fraction X1, where in a previous work C 2 was claimed to be located (1) The maximum for C'1 was not fully defined in this experiment, but it is clearly ahead of the C 2 activity It is not certain whether the C'1 activity was correlated with the electraophoretic *fraction* X2, as suggested in a previous paper (1) In repeated experiments the separation of C 2 and C'1 activities was always observed Thus it was also concluded that C'1 and C'2 are not localized to the same electrophoretic fraction From the figure it can be seen also that the C'4 maximum lies at the same level as β_{1C}, this was *a constant finding*

As previously mentioned, the serum in the preparatory experiments was concentrated 3–4 times, which led to a markedly unsharp separation *compared* with that obtained with the original serum This effect was manifest, *inter alia*, on complement activity which, as can be seen in Fig 2, exhibited considerable *spreading and overlapping* It cannot then be expected that any of the six eluates from the narrow electrophoretic region of about 10 mm can contain only one single *electrophoretic fraction* The concentration and further electrophoresis of the eluates showed that each of these in general contained at least two fractions

The preparative electrophoretic studies on concentrated serum thus

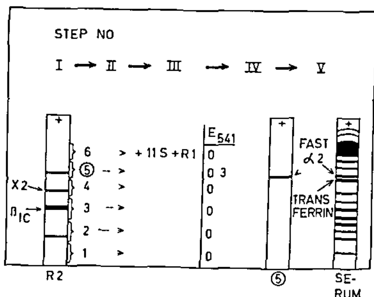


Fig 3

Scheme for isolation and identification of a protein fraction with C'1 activity

I Preparative electrophoresis of R2 reagent in starch gel
II Freezing thawing and elution of proteins from the gel segments 1 6
III Haemolytic activity in the eluates 1 6 measured as free haemoglobin at 541 mμ
IV Concentration of eluate No 5 (encircled)
V Electrophoresis in starch gel of concentrated eluate No 5 (encircled) Serum run parallelly as reference

showed that (*a*) the complement factors with the exception of C'3 could be localized to limited regions of the electropherogram, (*b*) the maximum of C'4 activity coincided with the β_{1C} band, (*c*) the maximum of C'2 activity with the electrophoretic fraction X1 and (*d*) the maximum of C'1 activity was found in front of fraction X1, but could not be definitely localized to the electrophoretic fraction X2

With the aim of obtaining more conclusive proof for the localization of C'1 and C'2 in the electropherogram, preparative electrophoretic separations of the complement reagents R1 and R2 were made R1 lacks C'1 and with the electrophoretic technique used R2 has only four or at the most five fractions altogether and lacks C'2 This greater "purity" in the starting material might be expected to improve the possibilities allotting the above complement factors in their proper electrophoretic bands

Preparative studies of R1 R1 was prepared by gel filtration and concentrated by freeze-thawing and preparative electrophoresis was carried out as above Only occasionally was C'2 activity demonstrated in the eluted fractions As with the electrophoresis of whole serum, activity was found in those fractions which were removed from the X1 region, but protein recovery was so low that, in spite of prolonged concentrations of these C'2-containing eluates, further electrophoresis yielded no visible bands

Preparative studies of R2 In a number of experiments in which C'1

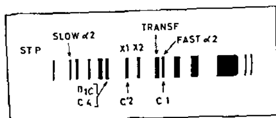


Fig 4

Schematic diagram of electropherogram of a human serum The localization of complement factors C'1 C2 C'4 and β_{1C} is shown

activity was measured in the eluates from electrophoretically separated R2, it was found that the fraction which contained the maximum of C'1 activity after concentration and further electrophoresis always lacked X2 The concentrated eluate always contained as a main fraction a band which corresponded in position to the fast α_2 of whole serum and sometimes also a weak band further towards the albumin In a few separations the author succeded in cutting out the fractions so that only one of them contained C'1 activity Concentration of this fraction and further electrophoresis resulted in only one visible band in the electropherogram, and this corresponded with the fast α_2 In Fig 3 the scheme of the stepwise isolation and identification of C'1 is illustrated After separation of the R2 reagent the gel was divided into six segments and each of the eluates from these segments was tested for C'1 activity As stated earlier, it was necessary to introduce the "11S" component and sometimes also β_{1C} in the immune haemolysis reaction Otherwise no C'1 activity could be demonstrated As "11S" reacts with the EA complex before C'1, it was considered convenient to introduce the different reactants to the test tubes in the order EA + 11S + eluate of gel + R1 + β_{1C} As seen from the figure, only the eluate from the segment No 5 contained C 1 Testing this eluate with the reagents R3 and R4 gave no haemolysis, even if 11S and or β_{1C} were added Subsequent electrophoresis of the concentrated eluate No 5 demonstrates a fraction corresponding to the fast α_2 region of whole serum

The results of the preparative studies with R1 and R2 can be summarized as follows (*a*) Studies with R1 reagents confirmed that C'2 was found in the region where X1 migrates (*b*) Experiments with the R2 reagent indicated that C'1 was localized to a single fraction which corresponded in position to the fast α_2 (Fig 4)

DISCUSSION

Preparative starch-gel electrophoresis was found to be less suitable for isolation and purification of complement factors Both recovery of protein and especially complement activities were unsatisfactory Whether the large loss of complement arises because this remains in the

residual 40 per cent of the protein that could not be eluted from the starch gel with the method used in this investigation (freeze-thawing) or whether it is due to direct inactivation is unclear. As regards the other elution methods mentioned above, it is the present authors experience that on electrophoresis the complement factors, with the exception of β_{1C}, are inactivated to an equal extent in polyvinylchloride as in starch gel. This method, like the electrodialysis method, is rather time-consuming as compared with the freeze-thawing method, and since the time factor is generally of great importance for the retention of the activity in complement isolation, it is hardly probable that the yield would have been larger But if it had been, the information as regards the localization of the components in the starch-gel electropherogram would probably not have been altered It is reasonable to assume that complement itself does not tolerate the electrophoretic procedure, since serum introduced into gel without electrophoresis and *kept at +4° C for 24 hours only lost about 50 per cent of the complement activity* The starch gel, as such, also has some specific effect on complement This view is supported by the fact that separation of serum in the presence of EDTA, which is not in itself complement-protecting, gave a reduced complement loss

Even if the separation method used is by no means satisfactory, it has, however, sufficed to throw further light on the hypothesis formulated in a previous study In this hypothesis it was postulated that (*a*) the electrophoretic fraction X1 contained C'2 and (*b*) the complement factor C'1 is included in the fraction X2 With regard to postulate (*a*), in the separation of whole serum the maximum activity of C'2 always corresponded with the electrophoretic fraction X1, which constitutes good support for the correctness of the hypothesis The fact that X1 was strongly heat-labile and disappeared on treating the serum with immune aggregate adds to the likelihood that C'2 is contained in the X2 fraction itself Further data in favour of this view will be presented in a future study of complement changes in connection with burns (20) In these investigations there was a good correlation between the C'2 titre and the magnitude of the X1 fraction

Point (*b*) in the hypothesis, on the other hand, *could not be verified* In experiments with serum C'1 was displaced in front of the X2 fraction In more conclusive experiments, performed with R2 reagent, C'1 was *localized to the fast α_2 region In experiments, after concentration* and electrophoresis of the C'1-containing eluate, there was only one fraction visible in the fast α_2 region This, however, does not mean that the *isolated fraction in the fast α_2 position* mainly consists of C'1 One reason for assuming that this band is inhomogenous is that no diminution of the band corresponding to the fast α_2 was visible after treatment of euglobulin with immune aggregate, in spite of the fact that this treatment decreased the C'1 titre by almost 100 per cent (1)

Apart from these considerations, the investigations have shown that

C'1 and C'2 can be localized to rather narrow zones of the starch-gel electropherogram It should be added that this is also true for C'4, which, however, seems to migrate together with β_{1C}

There remains the search for their exact relation to the electrophoretic bands in which they are found These results are, however, somewhat limited by the insufficient protein recovery and poor complement recovery in preparative starch-gel electrophoresis These circumstances could give rise to speculations whether the complement components could be hidden in other parts of the electropherogram Though not very reasonable such possibilities cannot be ruled out at present Anyhow in further studies the effect of the supporting medium on complement must be taken into consideration

Finally it was shown in the preceding paper (1) that X2 in the euglobulin serum fraction was heat-labile If it is assumed that C'3a is identical with β_{1C}, C'3b and C'3c have still to be localized and the possibility thus remains that X2 has some correlation to complement, namely through these two C'3 components Investigations on this question are in progress

There is at present no other electrophoretic method suitable for studies of complement components For example, with the immune electrophoretic method only β_{1C} can be detected with confidence (21) In addition to the basic importance of knowing the functions of the electrophoretic fractions, the different migration rates established for the complement components so far localized in starch gel may be of importance in future isolation and purification of these proteins

SUMMARY

By preparative starch-gel electrophoresis of serum and the serum euglobulin and pseudoglobulin fraction and the determination of the complement activity in the protein fractions eluted from the starch gel, it was shown that the complement factors C'1, C'2 and C'4 migrate at different rates C'4 was localized at a level corresponding to β_{1C}, concealed among the haptoglobins C'2 was found anodically to C'4 in the alpha beta region and C'1 anodically to transferrin on a level with the fast α_2 fraction of the serum Their relation to the electrophoretic fractions is discussed

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CHANGES IN THE HUMAN COMPLEMENT SYSTEM FOLLOWING BURN TRAUMA

By

K. E. FJELLSTRÖM and G. ARTURSON

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When a starch gel was used as supporting medium in electrophoresis, we observed in preliminary investigations of sera from burned patients, among other changes, an almost total disappearance of two fractions migrating cathodically to the transferrin.

It has been shown by *Fjellström* (1) that these two fractions are heat-labile, especially the slow-migrating one. In Hp group 1–1 and 2–1 sera according to the nomenclature of *Smithies* (2), the fast-migrating one contains a haptoglobin, but in Hp 2–2 sera which lacks haptoglobin in this region a stainable band is also found. With the exception of the haptoglobin in the fast moving band the functions of the proteins migrating in the region described were unknown. For this reason the slow-migrating one was provisionally called X1 and the fast-migrating X2 (1). Because of the heat lability a relation to complement was expected and later on it was showed that X1 probably contains the second complement component C'2 (3).

As there are no publications, as far as we know, on complement in burns, the observation that X1 and X2 were eliminated in sera from burns led to the investigation presented here.

The aims of this investigation were thus (a) to see if there are any changes of the complement system in serum from burned patients and if so, (b) to see if there is any correlation between such changes and the electrophoretic fractions. For comparison, changes in the serum protein pattern were also studied by means of paper electrophoretic method and alterations in the haptoglobin were noted.

MATERIAL AND METHODS

Patients

A total of ten patients was used for the investigation: (a) four severe burn cases with fatal outcomes, (b) three less severe burn cases who survived and (c) as controls three patients undergoing surgical interventions one of which also received clinical dextran.

Immune haemolysis

Amboceptor against Forssmann antigen was prepared by us according to the method of *Rapp* (4). The sensitization of sheep erythrocytes as well as the immune hemolysis procedure followed the principles outlined by *Kabat & Mayer* (5). In

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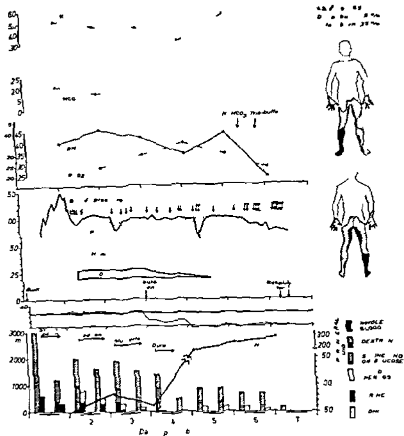


Fig 1

Case 1 Male aged 59 years Extent of burn total deep = 35 15 Died seven days post burn The figure shows potassium and acid base metabolism blood pressure pulse hematocrit rectal temperature parenteral fluid given urinary output and NPN (non protein nitrogen) Each arrow above the blood pressure curve indicates

↓ = 5 mg a oxine iv

● = *2 mg noradrenalin*

Case 1 Male aged 59 years Extent of burn total deep = 35 15 per cent Survival time 7 days 90 per cent mortality risk according to *Bull & Fischer* (12) Clothes set alight by exploding petrol Hospitalized immediately in bad condition with low arterial blood pressure and hemoconcentration Fluid replacement and other forms of treatment are illustrated in Fig 1 On the second day acute decrease of blood pressure ... the
patient was ... tion
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temperature was increased to about 32 (Yet the patient died on the 7th day post burn in a state of anuria with high NPN values decreasing arterial blood pressure hyperpotassemia and metabolic acidosis which was not compensated by hyperventilation At autopsy the following changes were found fatty infiltration and degeneration of areas of liver cells changes in the kidney of the lower nephron nephrosis type and edema of the lungs and brain

TABLE 1

The Titers of C 1 C 2 and C 3 in five Randomly Chosen Fresh Normal Sera Given in 50 per cent Hemolysis Units Every Serum Is Titrated Twice (Numerals 1 and 2)

Normal Serum no	C 1		C 2		C 3	
	1	2	1	2	1	2
I	1730	1684	470	484	190	200
II	1939	1830	500	400	285	307
III	1830	1850	400	380	258	242
IV	1882	1939	363	363	200	210
V	1790	1830	533	500	222	242

titrations of the complement components C 1 C 2 C 3 and C 4 the four complement reagents R1, R2 R3 and R4 were used R1 and R2 were prepared by means of gel filtration (6) and R3 and R4 by treating fresh normal human serum with zymosan and hydrazine respectively (7, 8) In testing the sera from burned patients for anti complementary effect 0 1 ml of these sera diluted 1 5 were added to a serial dilu tion of normal serum None of the burn sera investigated did decrease the degree of hemolysis of the normal serum thus allowing the conclusion that sera from burned patients were not anticomplementary

The values of the titres were calculated from the *von Krogh* formula (9) after plotting the percentage hemolysis values from the different dilution on a double logaritmic graph paper

In the case of severe burns the titrations of the four complement components were performed daily on fresh serum The values were expressed as a percentage of the values in a simultaneously titrated serum from a different healthy blood donor each day thus avoiding the possibility of an abnormal control serum from a single donor Further this was considered to be correct as it is known that the normal range of titres of the four complement components is rather narrow This is also true for the immune hemolysis technique used by the present authors as demon strated in Table 1 where the results of titration of C 1 C 2 and C 3 of five ran domly chosen normal sera are given

In the less severe cases preliminary titrations showed small changes in comple ment titres For that reason and in order to increase the accuracy of the method all sera from one and the same patient were stored at —20° C together with three normal sera All these samples were then analyzed at the same time *i e* with the same sheep blood cells and complement reagents during the same day

Electrophoresis

Paper electrophoresis was carried out using a veronal buffer of pH 8 6 and ionic strength of 0 1 Starch gel electrophoresis was performed according to *Smithies* (2) in *Poulik s* modification (10) with a discontinous system of buffers as described in detail earlier (1)

Quantitative Methods

The total serum proteins were determined by the Kjeldahl method Double deter minations were made and the mean noted The total amount of haptoglobin in serum was determined according to *Smith & Owen* (11)

RESULTS

(a) *Severe Cases of Burns*

Three of the four severely burned patients in this study died on the second or third day after the burn trauma In the fourth case, however, the serum complement could be investigated for seven days post burn A short report on this patient is given below

Fig 1

Case 1 Male aged 59 years Extent of burn total deep = 35/15 Died seven days post burn The figure shows potassium and acid base metabolism blood pressure pulse hematocrit rectal temperature parenteral fluid given urinary output and NPN (non protein nitrogen) Each arrow above the blood pressure curve indicates

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ampu[illegible] above the right knee was performed and at the same time the body temperature was decreased to about 32° C. Yet the patient died on the 7th day post burn in a state of anuria with high NPN values decreasing arterial blood pressure hyperpotassemia and metabolic acidosis which was not compensated by hyperventilation At autopsy the following changes were found fatty infiltration and degeneration of areas of liver cells changes in the kidneys of the lower nephron nephrosis type and edema of the lungs and brain

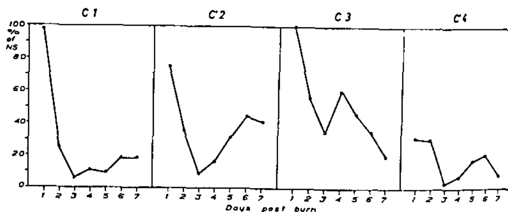


Fig 2

Changes of complement in Case 1

Abscissa: days post burn
Ordinate: titers of the complement components C′1, C′2, C′3 and C′4 expressed in per cent of the titers in a simultaneously titrated normal serum

TABLE 2

The Complement Level of Cases 2–4 During the first Day after Admission The Values Are Given as Percentage of the Titer of a Simultaneously Titrated Normal Serum

Cases	C′1	C′2	C′3	C′4
Case 2	5		63	8
Case 3	3		6	2
Case 4	8	30	52	21

In *Fig 2* are shown the changes in the serum complement following the burn trauma in Case 1 Three days after the burn the lowest titres were reached and C′1, C′2 and C′4 all decreased to values as low as 10 per cent of those of a normal serum but there was a less pronounced decrease of the C′3 titre The low values remained until death The tendency shown in the figure to increasing values of the complement components from the fourth day after the burn is insignificant This rapid initial fall, illustrated in Fig 2, of all the complement components was also found in the other three fatal cases (see Table 2)

(b) *Moderate Cases of Burns*

In the moderate cases of burn trauma, on the other hand, the decrease of serum complement was slight and in the least burned patients no decrease at all was noticed during the first few days after trauma Two of these surviving patients were followed several weeks and it was found that at the end of the first week the titre of the complement components began to rise and in the second week values were reached as high as two to three times that of the complement *level in normal*

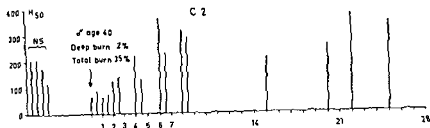


Fig 3

Case 5 The course of C2 titer during the four week period post burn

Abscissa to the left in the diagram the C'2 titers of four normal sera (NS) and to the right the C'2 titer of Case 2

Ordinate C'2 titer in 50 per centage hemolysis units (H_{50}) Every bar represents one titration

sera These high titres remained in the fifth week after the trauma, at a time when the burn wounds were healed The following patients are cases in this group

Case 5 Male aged 40 years Received second degree scalds of the arms legs and the back from hot lye Extent of burn total/deep = 35/2 Mortality risk 50 per cent Hospitalized immediately treated with exposure and penicillin Intravenous fluid replacement with dextran and electrolytes during the first two days post burn From the third day onward treated by mouth Moderate clinical signs of infection during the second week post burn with invariable growth of penicillinase producing penicillin resistant staphylococcus aureus in the wounds Four weeks after

[illegible] e arms and legs The course of

In Fig 3 are shown the changes in the C'2 component only (Case 5) but similar changes were also found in the other complement components

As this group of patients had predominantly superficial lesions and many blisters, it was considered to be of some interest to know the degree of leakage of complement into the blisters In two of the patients therefore the C'2 and C'4 titres were estimated in the blisters in the period between the second and sixth days after the trauma It was found that the titres of these components on the second day were about half those of the patients serum and when the titres in the serum gradually increased, the titres in the blisters decreased

(c) *Control Cases*

To see if there were any changes in complement following surgical trauma, two patients who had undergone open-heart surgery were investigated for a period of three days after the day of operation The only changes found were very slight decreases of C'2 and C'4 during the first day after operation in one of the patients But as they received

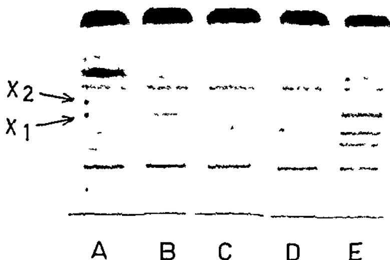


Fig 4

Case 5 A photograph of the starch gel electrophoretic picture of the patients serum compared with a normal serum

(A) Normal serum with hemoglobin added
(B) The same serum without hemoglobin
(C) Case 5 18 hours after the burn trauma
(D) Case 5 36 hours after the burn trauma
(F) Case 5 8 days after the burn trauma

large amounts of donor blood, the changes in C'2 and C'4 were hard to judge

Infusions of dextran in man is reported not to interfere with complement (13) Nevertheless one patient undergoing reconstructive face surgery received 1000 ml of clinical dextran (Rheomacrodex ®) intravenously The serum from this patient did not show any changes at all in the titre of the four complement components for three days following the infusion

The results of the starch-gel electrophoretic investigations are reproduced in Figs 4 and 5 In Fig 4 a photograph of the starch-gel electrophoretic picture of serum from a moderate case of burning (Case 5) is seen The X1 fraction is not visible in the two-day period after the trauma, when the C'2 titre was decreased by about 50 per cent Eight days after the burn trauma there is a remarkable increase of the X1 fraction, at which time the C'2 titre was also largely increased Fig 5 illustrates the electrophoretic changes in moderate cases of burn trauma observated for four weeks The starch gel electropherogram shows that from the end of the first week after the trauma there is an increase of several electrophoretic fractions, especially the haptoglobins But one of the most striking features is the very large increase of X1 At the same time the C'2 titer increased two to three times

The quantitative changes in haptoglobin and other serum proteins from Case 5 are illustrated in Fig 6 It is seen that the haptoglobins are

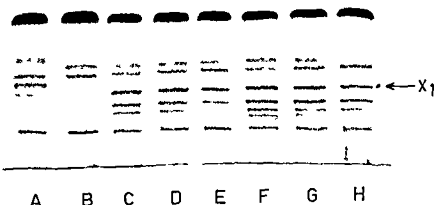


Fig 5
Case 5 and 6
(A) Normal Hp 1 1 serum with hemoglobin added
(B) The same serum without hemoglobin
(C) (D) and (E) is from *Case* 5 14 19 and 24 days post burn
(F) (G) and (H) is from *Case* 6 14 19 and 24 days after burn

already elevated from first day after trauma The highest values are reached about one week post burn and then gradually decrease towards normal The total protein concentration was initially very low and was nearly normalized after about three weeks This decrease was largely due to loss of albumin and in some extent also to γ globulin decrease while α_1 α and β globulins were slightly elevated

The results can thus be summarized as follows

(1) In cases of severe burns there is a pronounced decrease of the titres of the complement components in blood from the first day after trauma reaching a level of about 10 per cent of normal on the third day

(2) In cases with a fatal outcome this very low complement level remains until death

(3) In moderate cases of trauma the decrease in complement titre is slight or nil

(4) Whether or not there is an initial decrease the complement titres in the moderate cases reach a level two to three times higher than that in simultaneously titrated normal sera This increase appears at the end of the first week and still persists into the beginning of the fifth week

(5) The starch gel electrophoretic investigations have shown that in severely burned patients the X1 fraction was almost completely absent In moderate cases on the other hand there was usually only a slight decrease of X1 during the first few days after the burn and from the end of the first week X1 was largely increased

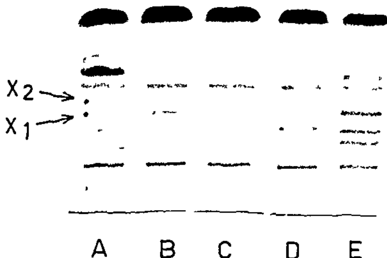


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DISCUSSION

There are some objections to titration of complement components with use of complement reagents concerning the theoretical background (5). Also the accuracy of the method must be considered low. If however the titrations are performed on the same day with the same population of sensitized erythrocytes and the same set of reagents the mean error has been reported to be not more than ± 5 per cent (14). These criteria were fulfilled in the cases of moderate burns but not in the cases of severe burns. Yet in the latter cases the changes of complement level were so marked that they must be accepted to be significant as more as no anticomplementary effect of the burned patients sera could be detected.

1. *The decrease of complement* titer in the severe cases can certainly have many explanations but only the following will be discussed: (a) leakage from the blood stream due to increased capillary permeability, (b) interaction between complement and dextran, (c) fixation of complement to antigen antibody complexes, (d) infection and (e) decreased formation or increased breakdown of complement.

(a) *Increased Capillary Permeability*

This is at first sight one of the most reasonable explanations. It was found by *Arturson* (15) that the degree of capillary permeability in burned areas is correlated to the severity of the burn trauma. In extensive burns the blood lymph barrier in non burned parts of the body is also more leaky than normal but this however does not apply to proteins, not even to albumin (15) which is one of the smaller protein molecules of serum.

The complement components are non dialyzable and are doubtless *proteins* (5) but their molecular size and shape are unknown. There are some evidence that they are globulins and it is therefore also possible that at least some of them have a molecular size larger than that of the albumin molecule. As the albumin content in serum even from severe cases of burns is found as a rule to be about 40–60 per cent of the normal range (16, 17) the 90 per cent decrease of complement cannot be explained as being due only to leakage through increased capillary permeability. Furthermore as α_1, α_2 and β globulins studied by the paper electrophoretic method increases during the first week a selective loss of complement components due to increased capillary permeability is not very likely. Finally it must be stated that in cases of second degree burns the leakage due to increased capillary permeability is more pronounced than in cases with third degree burns. Thus Case 1 in this study had primarily deep burns (third degree) and Case 5, 6 and the other moderate cases rather the second degree type of burn. From the standpoint of the leakage theory the severe cases should have had less decrease of complement than the moderate cases.

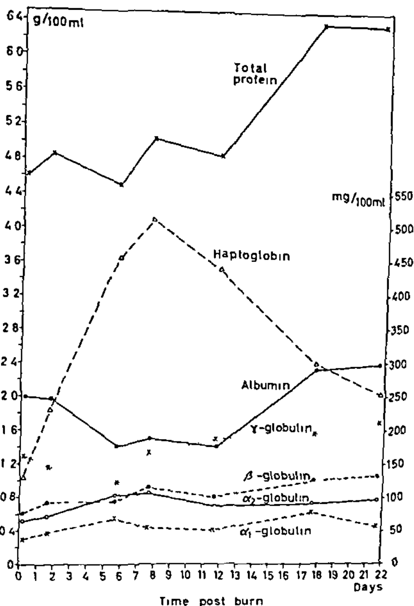


Fig 6

Case 6 Changes in plasma proteins during the first three weeks post burn
Left ordinate protein concentration in gram 100 ml
Right ordinate haptoglobin concentration in mg 100 ml

(6) Paper electrophoretic investigations and estimations of haptoglobin concentration in cases of moderate burns confirmed earlier known facts that the decrease in total protein is mainly caused by albumin loss The α- and β-globulins were slightly increased and the increase in haptoglobins was even more pronounced

(d) *Infection*

Infection as a cause of low complement titre in burns could also be questioned because such changes are often observed in infectious diseases (26 27 28) *Ecker et al* (28) have shown that patients with epidemic (meningococcal) meningitis and subacute bacterial endocarditis have very low plasma complement Especially C4 and/or C1 and C2 were decreased when the illness was in a severe phase However in some acute infections elevation of complement titre has also been reported (28) Susceptibility to infection is increased following thermal trauma and septicemia is a common cause of death between the second and seventh day after admission (29) but there were no signs of septicemia in any of the patients in the present material Some of the complement loss may in fact be explained by infection Thus it has been shown that there is an antibody response in burned patients against the bacilli colonising the burn wounds (30) and that the endotoxins decrease the complement level in experimental animals only in the presence of natural antibodies (31)

(e) *Decreased Formation or Increased Breakdown of Complement*

Other explanations of a low complement titre could be a decreased formation of one or more of the complement components or increased metabolically controlled breakdown This is known to be true in the case of albumin Thus *Birke et al* (32) and *Davies et al* (17) found that in burns the catabolism of albumin is increased with a maximum on the third day after the trauma It is not known if this is also true of the globulins The increased values of these latter proteins already found on the second day after burning make such a possibility unlikely

To sum up the causes of the low complement titres in the acute phase of severe burns may thus be manifold At any rate it is reasonable to assume that part of this complement loss is due to leakage from the blood on account of increased capillary permeability This cannot however explain the whole loss of about 90 per cent of the complement The suggested explanations for the rest of the loss are somewhat obscure as well Inactivation from such causes as dextran infusion is probably unimportant Decreased formation or increased breakdown of complement as well as fixation of complement to immune complexes cannot be ruled out for the present The uncertainty in explaining the complement loss points to a need of more experimental work and first of all of the question whether complement are fixed to thermally damaged cells or not What so else it might be expected that further complement studies in this field may contribute to increased knowledge of the pathogenesis of the post burn period

2 The reason for *the increase of serum complement* from the end of the first week after the trauma in mild cases of burns is also unclear Increased values of complement have been observed for example in

The leakage of protein into the urine was very slight or none at all in all the burned patients in the present material. It has also been shown by *Lange & Wenk* (18) that even in severe proteinuria the complement titer in the urine is very low and independent of the titer in the plasma.

(b) *Interaction between Complement and Dextran*

The ability of dextran to combine with properdin in paroxysmal nocturnal hemoglobinuria has been discussed (13). As the burned patients in the present material as a rule received dextran, it was checked whether or not dextran could be responsible for some of the complement loss. This was done by giving a patient who had undergone a surgical operation the same type of dextran in an amount almost similar to that given to moderately burned patients. No decrease of complement titre could be found during the first three days following the dextran infusion. Thus dextran does not play an important part in the low complement titres in cases of severe burns.

(c) *Fixation of Complement in Antigen/Antibody Reactions*

Some immune mechanism operating in burns has previously been discussed. Thus *Feodorov et al.* (19) and *Pavkova et al.* (20, 21) found antibodies in sera from burned patients directed against burned skin. According to the latter authors, the antibodies were detectable in the blood between the fourth and eighth days after burning. The titer reached a peak about the fifteenth day. Then the antibody titer gradually declined but in the convalescence period a new increase was noticed. Similar observations have also been reported by *Rosenthal et al.* (22, 23). They found "toxic substances" in sera from recently burned patients which they called cytotoxins and cytolysins. In sera from patients who recovered they could also detect antibodies (antitoxins) directed against the above mentioned cytotoxins and cytolysins.

Another disease with a supposed immune mechanism in its pathogenesis is acute glomerulonephritis, in which changes in the complement system have been studied (24). In this disease the complement decrease parallels the clinical signs of outbreak, *i.e.* the proteinuria and impaired kidney function, and, when recovery sets in, the complement titre returns to a normal level. If it is assumed that complement is consumed in antigen/antibody reactions *in vivo*, and evidences for this do exist, (25) a similar mechanism as in the nephritic patients may operate in cases of burns. The low complement titres at the end of the first week in cases of severe burns could then be due, at least partly, to fixation of complement to antigen/antibody complexes, as it is known that antibodies against burned skin are detectable in the blood at this time (20, 21).

BRIEF REPORT

INFECTION OF NATURALLY INSUSCEPTIBLE CELLS WITH DNA OF POLYOMA AND SV_{40} VIRUSES

By *Hans Diderholm* and *Tore Wesslén*

DNA of polyoma and SV_{40} viruses

Methods Polyoma virus was grown in mouse embryo cell cultures; SV_{40} in cultures of kidney cells from the African green monkey, *Cercopithecus aethiops* Both viruses were concentrated 100 fold by dialysis against polyethylene glycol powder (PEG 20M) followed by ultracentrifugation After concentration, the suspensions of polyoma virus contained 2048–16384 HA units per 0 5 ml The titers of SV_{40} concentrates were $10^{8.5-9}$ $TCID_{50}$ per ml

To 4–6 ml quantities of virus suspension, equal amounts of phenol saturated with 0 02 *M* phosphate buffer pH 7 2 containing 0 01 *M* versenate were added The mixture was shaken vigorously by hand for 4 minutes at room temperature and then centrifuged for 3 minutes at 4000 rpm The upper phase was removed and again treated

... prepared similarly from uninfected mouse embryo and monkey kidney cultures

The extracts were diluted in equal amounts of 0 08 *M* phosphate buffer pH 7 8 containing 1 2 *M* NaCl and were added to mouse embryo monkey (*Cercopithecus*), swine and calf kidney cultures which had been rinsed in Hanks' ...
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Sample
ma viru
f SV_{40} by inoculating in
rabbit antisera to polyom
to ensure that the effect ... virus produced and not to surviving infectious DNA

... mouse embryo, monkey ... studied The amount of ... titrations in tubes with ... cells

Results Samples taken from all four types of cultures inoculated with extracts from polyoma or SV_{40} viruses were found to produce cytopathic changes identical with those caused by the intact virus These changes could be ... addition of antiserum ...

... in kidney cul ... not show any

This investigation was supported by grants from the Swedish Cancer Society
Received 14 ix 63 from the *Institute of Virology University of Uppsala Uppsala Sweden*

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FROM THE DEPARTMENT OF GYNAECOLOGY, KOMMUNEHOSPITALET, COPENHAGEN (HEAD FRITZ FUCHS, M.D.) AND
THE NEUROPATHOLOGICAL LABORATORY, RIGSHOSPITALET, COPENHAGEN (HEAD ERNA CHRISTENSEN, M.D.)

OLIGOHYDRAMNIOS AND URINARY MALFORMATIONS IN EARLY HUMAN PREGNANCY

By

GORM WAGNER and INGE TYGSTRUP

Received 31 i 63

The formation of amniotic fluid has long been under discussion. any cases of oligohydramnios in connection with malformations of e foetal urinary tract have been described (1, 2, 3, 4, 5, 10, 12, 15) d these cases have given support to the supposition that the foetal dneys play an important part in the formation of amniotic fluid owever, to our knowledge oligohydramnios has only been described the last trimester of pregnancy In the present paper we shall report case of oligohydramnios connected with urinary tract malformations a 16 weeks old foetus

CASE REPORT

A 34 year old woman pregnant for the eighth time was admitted for legal abor n and sterilization in December 1960
Her past history revealed congenital syphilis with a positive Wassermann test ntil her fifth year Ever since it had been negative on several occasions
Apart from an uncomplicated appendectomy at the age of 16 she had not been cated in hospitals
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Discussion The studies on the propagation of polyoma and SV_{40} viruses indicate that monkey, swine and calf cells are naturally insusceptible to polyoma virus whereas mouse, swine and calf cells are naturally insusceptible to SV_{40}

The present results indicate however that phenol extracts derived from cultures infected with polyoma or SV_{40} viruses are capable of infecting these cultures The appearance of the cytopathic changes obtained in passage cultures and the fact that they were prevented by the addition of antiserum are strong evidence that polyoma and SV_{40} viruses had been formed The infectious properties of the extracts were inhibited by DNase which supports the assumption that the infectious substance is DNA Thus it appears very likely that the DNA of polyoma and SV_{40} viruses is capable of infecting not only naturally susceptible cells as reported before (4 5 6 7), but also cells which seem to be naturally insusceptible to the intact virus

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...rom placenta was mixed into it ... the measuring cylinder some blood ... The total volume thus measured was 35 ml The

haemoglobin content was 20 per cent and the amount of amniotic fluid therefore was estimated to 25–30 ml which is much less than usually found at this stage

Because of this reduced volumen of amniotic fluid an autopsy of the foetus was carried out

Macroscopic Examination

The placenta measured 8 × 7 cm and weighed 86 g According to Potter this is a normal weight (11) The umbilical cord inserted centrally and contained three vessels The membranes were not examined for signs of amnion nodosum The foetus measured 112 mm (CR) and weighed 95 g External examination revealed malformation of the genital region which presented itself as an elevated oval area without openings No anus was seen The neck and thoracic organs were normal The osseous system and central nervous system were without abnormalities The organs in the abdominal cavity were normally placed, rectum ended with a slight dilatation behind the urinary bladder The rest of the gut, the stomach, gall bladder, pancreas, spleen, adrenals and large vessels were normal The urinary system was malformed In the left side no kidney or ureter could be found In the right side a ureter-like band could be followed from the bladder to the caudal surface of the adrenal, where an irregular piece of tissue, possibly kidney tissue, was demonstrated The dilated urinary bladder contained 2 ml of a clear yellow fluid It was not possible to find any ureteral openings inside the bladder No urethra nor prostate could be seen The testes were lying on the posterior wall of the peritoneal cavity

Microscopic Examination

The placenta and the umbilical cord showed normal conditions The possible kidney tissue from the right side was only a fourth of the size of the adrenal gland The tissue was built like a kidney with a narrow cortex containing scattered glomeruli (Fig 2) The tubular structures, too, were lying scattered in the mesenchymal connective tissue Normal mucous membrane of the pelvis was seen in a small area The bladder had a normal wall Tissue from the bottom of the bladder revealed no prostatic tissue

The final diagnoses were aplasia renis et ureteris sinistris—hypoplasia renis dextris—atresia urethrae et ani

DISCUSSION

In a recent publication *Wagner & Fuchs* (1962) have reported the amount of amniotic fluid in the first half of human pregnancy In the 15th week of pregnancy an average of 125 ml of amniotic fluid was found For each week before the 15th week (until the 11th week) the amount is about 25 ml lower and for each week after it is about 50 ml more than 125 ml

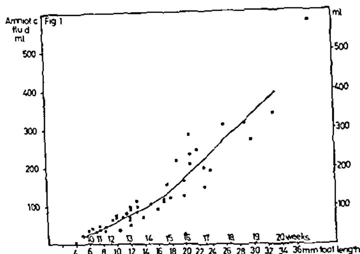


Fig 1

Volume of amniotic fluid against foetal foot length The curve indicates the average in 45 normal subjects The triangle indicates the foetus with urinary tract malformations (From Wagner & Fuchs 1962)

Fig 2

Section of the right kidney Scattered glomeruli and tubuli To the right cortex of the normal adrenal gland Haem Eo × 100

Fig 1 shows an average curve from normal subjects The amount of fluid from the present case is indicated by the triangle and it is seen that it only represents about 20 per cent of the average in 15th to 16th week of gestation

In Table 1 the data concerning the age of the present pregnancy are shown

TABLE 1

	Weeks of gestation
Period from last menstrual flow	15–16
Pelvic examination	12–13
Foot length of foetus 20 mm corresponds to	16
Crown rump length 112 mm corresponds to	15–16
Weight 95 g corresponds to	15
Volume of amniotic fluid 25–30 ml corresponds to	11

There is pathological as well as physiological evidence of function of the foetal kidney in late pregnancy In 1872 *Gusserow* reported 10 cases with dilatation of the urinary bladder due to obstruction The amount of urine in the bladder varied from 250 to 2000 ml From the literature he collected 18 more cases, including the first described case of oligo hydramnios by *Portal* (1671) who concluded that the amniotic fluid was formed by the foetal urine

In 1932 *Neale* published one case of dilatation of the bladder due to urethral valvula *Wells & Bell* described in 1946 four cases of dilatation of the upper part of the urinary tract from an autopsy material of 52 stillborn children with spina bifida Hindrance of free urine flow could be demonstrated in two of these cases

In 1951 *Kinnunen* published 15 cases of urinary tract malformation resulting in stasis In an unpublished material of 600 consecutive post-mortem examinations of children who died perinatally *Tygstrup* (13) found 2 cases of urethral atresia combined with dilatation of the bladder and ureteres Of special interest in connection with the present case is the unusual finding by *King* who in 1837 described a case with impervious urethra, dilated ureter and rupture of the urinary bladder in a four month old male foetus The bladder was estimated to have contained "rather more than half a pint of water

To the question whether the early foetal kidney is producing urine and thus (if the urine is passed) participating in the formation of the amniotic fluid or is a non functioning state until late gestation it should be mentioned that *Wagner & Fuchs* (1962) when measuring the foetuses in their series found that quite a number of foetuses between 13 and 20 weeks passed urine when slight pressure was applied to the lower abdomen

Several clinical observations of the possible relationship of the

amount of amniotic fluid and the kidney function have been published. In 1908 *Hauch* published one case of bilateral renal agenesia in a one month premature child with at most 30 ml of amniotic fluid. *Bardram* (1930) found two cases of bilateral agenesis in a material of 60 new born children with kidney malformations. The first had probably, the second certainly oligohydramnios. Further he found that in many of the remaining children there was probability of decreased volume of amniotic fluid as judged from the hospital records. In 1959 *Jeffcoate & Scott* made a survey of the literature on renal agenesis and found that of the 300 published cases oligohydramnios could be demonstrated with certainty in 100 cases. *Potter* (1946) found 20 cases of bilateral agenesis in 5000 consecutive autopsies on newborn children. She expected to find oligohydramnios, but the relation between these two conditions could not be verified in this publication. However, in her textbook from 1961 *Potter* writes that "among 49 cases in which post mortem examinations were made at the Chicago Lying in Hospital on fetuses with bilateral agenesis, we have never been able to find an obstetrician who actually saw amniotic fluid in such a pregnancy.

That the foetus does produce urine before birth is certain as the new born often starts its life by passing urine. It may even be so that the amount of urine produced in utero is relatively greater than later in life, as the kidney before birth (as judged by the character of the first voided urine) is not under control of the anti diuretic hormone (*Mc Cance & Widdowson* 1953).

It seems that even in early pregnancy the foetal kidney is in function and that the foetal urine is participating in the formation of the amniotic fluid.

SUMMARY

In a 16 weeks old pregnancy that was interrupted by hysterotomy the ovum contained very little amniotic fluid. Autopsy of the foetus showed severe malformation of the urinary tract including urethral atresia. Oligohydramnios has never before been described in early pregnancy and the case supports the theory that the foetal urine takes part in the production of amniotic fluid and that this is the case even in early pregnancy.

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CULTURE OF HUMAN FIBROBLASTS ON GLASS PLATES

By

S BERGMAN

Received 7 iii 63

In the fields of virology, genetics and biochemistry there is an increasing need for growing and surviving cells in a good condition Serum is necessary for the growth of most cells but serum may be toxic and attack the cells in many ways (*Puck et al* 1958, *Paul* 1960, *Saksela et al* 1960, *Terasaki & Chamberlain* 1962 and others)

The reaction of cells to their environments has long been used in the investigation of the effect of different substances upon the cell Many methods have been devised to measure the growth amount or death of cells Monolayer cell culture on glass plates in Hellenthal cuvettes (*Bergman* 1959) provides a simple method for genetic (*Bergman et al* 1960) and virological work (*Bergman & Jonsson* 1963)

This paper is concerned with the culture of human fibroblasts and various factors influencing their growth

MATERIAL AND METHODS

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This procedure ensures a fairly uniform examination of the entire cell surface Twenty fields of vision were counted on each glass and 2 glasses were used for each test In the event of substantial differences between the numbers found in the different fields of a given glass the later was discarded The ratio between the total number of mitoses on 20 fields of vision and the mean value of cells on 20 fields of vision per glass was taken as the mitotic index

Primary Culture of Human Fibroblasts

Cells were taken from the skin of 1–4 month old human embryos obtained on therapeutic abortion The skin was cut into small pieces treated with trypsin in 0 1 per cent trypure Novo for 1 hour and then seeded in Carrel flasks which were stored at 37° C. The medium used was Parker 199 with 20 per cent pooled uninactivated human serum The subsequent growth in the flasks was treated with

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MATERIAL AND METHODS

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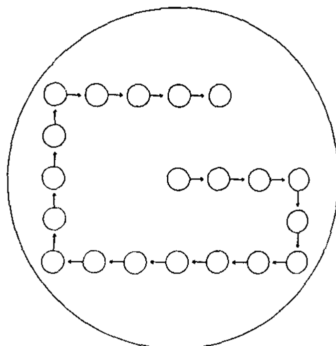


Fig 1

trypsin washed counted in a Buerker haemocytometer and diluted to desired concentration in Parker 199 with 2 per cent pooled uninactivated calf serum Hank's solution (the cells were fastened at 37° C) To avoid contamination all the cells were studied for chromosomes and established cell lines were not cultured in the same department

Time Allowed for Attachment of Fibroblasts on Glass Plates

Experiments with varying periods of 2, 4, 6 and 16 hours showed 2 hours to be sufficient for optimal attachment of the cells

Comparative experiments with attachment after suspension in Parker 199 with 2 per cent calf serum or Hank's solution showed no differences in cell attachment during the first 4 hours For practical reasons this time was therefore chosen Attachment of the cells in Hank's solution for 16 hours "injures" the cells and no growth occurs This applies to inocula of 50,000, 100,000 and 200,000 cells ml

Significance of Number of Cells in Inoculum

The number of mitoses and the attachment of the cells were studied for any variation with number of cells in the inoculum (Fig 2)

RESULTS

As can be seen in Fig 2 the highest number of mitoses was obtained with 200,000 cells/ml which gave a dense cellular carpet on the glass

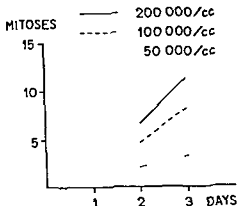


Fig 2

Significance of number of cells in inoculum Attachment of cells in Parker 199 with 2 per cent calf serum for 4 hours

When larger inocula were used growth was difficult to judge and involved the risk of loosening of cells Smaller numbers of cells gave sparse outgrowth

Variation in Attachment of Cells in Different Flasks Seeded with Cells from same Foetus and from Different Foetuses

The cells were taken from flasks in which the medium had been changed the previous day The cells were suspended in Parker 199 with 2 per cent calf serum and allowed 4 hours attachment The number of cells in the inoculum was 200,000 ml and the mean number of the 20 fields of vision counted on each glass plate is given in Table 1

TABLE 1

Variat on n Cell Att ch ... per cent Calf ... d Growth in ... Mean Value ... ks (I and II) ... d

Foetus	A		B		C		D	
Flask	I	II	I	II	I	II	I	II
Obj gl 1	25	30	21	23	26	31	26	24
Obj gl 2	28	29	23	27	23	29	24	23
Obj gl 3	28	28	21	26	28	28	24	20
Obj gl 4	26	24	27	25	22	22	25	28
4 obj gl 20 fields of vision of each	107	111	92	101	99	110	99	95
Largest difference between obj gl	3	6	6	4	6	9	2	8

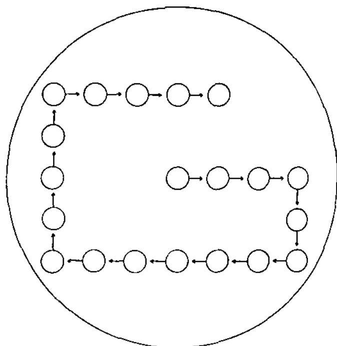


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TABLE 3

Effect of Change of Medium of Primary Culture on Number of Mitoses on Object Glass

	I	Mean value of 4 obj gl counted on 2 days outgrowth	
		II	III
Foetus 19/5 Seeded with 200 000 c/ml	1 day	17.5	5.5
	3 day	17	2.5
	4 day	19	2.75
Foetus 1/6 Seeded with 200 000 c/ml	1 day	26.5	9.5
	2 day	26	6.5
	3 day	23.5	4

I A varying number of days before trypsination and transfer to obj gl
II Cell density Average of 20 fields of vision/obj gl
III Total mitoses in 20 fields of vision/obj gl

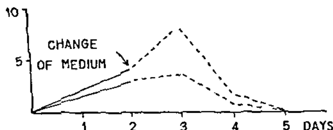


Fig 3

Effect of change of medium in cuvette on number of mitoses (20 fields of vision)

In an attempt to study the effect, if any, of the change of medium in the cuvette on the growth experiments were carried out with the same culture and the same medium in 2 cuvettes, in one of which the medium was changed on the second day (Fig 3)

Fig 3 shows that the change of medium on the 2nd day increased the number of mitoses For practical reasons including the consumption of medium, however, it was decided not to change the medium in the cuvettes in subsequent experiments

Effect of Composition of Medium on Cell Growth

In media employed for routine studies we used 20 per cent human serum with Parker 199 In order to test whether this amount of serum was sufficient or whether it could be reduced, experiments were made with preparations from the same foetus and with the object glasses placed in the cuvettes with varying amounts (40 20, 10, 5 and 0 per cent, respectively) of serum (Fig 4)

RFSULTS

In this series (Table 1) the cells of varying age were taken from foetuses (A 22 days old; B 12 d; C 7 d; D 3 d) Within these age limits the cells showed no substantial differences in attachment or differences between the mean value found on the glasses Neither were any substantial differences found between foetuses or different flasks of cultures of material from one and the same foetus

Number of Fields of Vision and Differences Between Different Examiners.

In this experiment the number of cells and number of mitoses were counted separately (Table 2) by two examiners (K and B)

TABLE 2

Comparison Between Counts of Cells and Mitoses by two Persons (K and B)

Obj glass Number	K		B	
	Cells	Mitoses	Cells	Mitoses
1059	20	6	22	6
1111	18	2	20	3
1118	23	8	20	9

RESULTS

It is clear from Table 2 that the differences between the counts by the examiners were well within acceptable limits

Effect of Change of Medium on Primary Cultures and Growth in Cuvettes

The medium in the Carrel flasks containing preparations from 2 foetuses (C and D) was changed a varying number of days before transfer to the object glasses The same fibroblasts were cultured in Carrel flasks for a varying period before they were transferred to the object glasses The object glasses were placed in the same cuvette (Table 3)

RESULTS

It is clear from Table 3 that the highest number of mitoses was obtained when the medium was changed in the flasks the day before trypsination The number of cells, however, *did not appear to vary with the* interval between change of medium and transfer of the culture to the object glass

TABLE 3

Effect of Change of Medium of Primary Culture on Number of Mitoses on Object Glass

	I	Mean value of 1 obj gl counted on 2 days outgrowth II	III
Foetus 19/5 Seeded with 200 000 c/ml	1 day	17 5	5 5
	3 day	17	2 5
	4 day	19	2 75
Foetus 1/6 Seeded with 200 000 c/ml	1 day	26 5	9 5
	2 day	26	6 5
	3 day	23 5	4

I A varying number of days before trypsination and transfer to obj gl
II Cell density Average of 20 fields of vision/obj gl
III Total mitoses in 20 fields of vision/obj gl

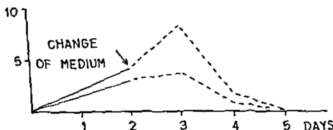


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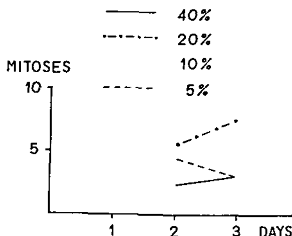
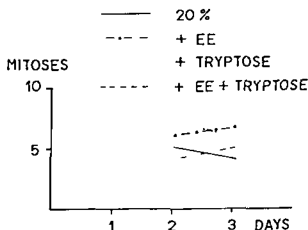


Fig 4

Effect of variation of amount of serum on number of mitoses (20 fields of vi

TABLE 4

	Mitotic index 2nd day	
20 % human serum	5 75	26 5
20 % human serum + EE	10 5	22 5
20 % human serum + tryptose	7 5	27
20 % human serum + tryptose + EE	9 75	25



Figs 5 and Table 4

Effect of addition of tryptose and embryonal extract to medium on number mitoses (Fig 5) and on mitotic index (Table 4)

RESULTS

When serum was not added, no mitoses occurred and the cells did n appear to be healthy when they were stained 2 days later The highe number of *mitoses* was *obtained with 20 per cent* serum

The addition of tryptose phosphate broth (Difco) or embryonal extract (EE) from eggs was studied (Fig 5 and Table 4) In Table 4 the mitotic index (see p 3) has been used

RESULTS

Addition of these two growth factors increased the mitotic index It is possible that EE had a somewhat better effect than tryptose A combination of the two appears to offer no greater advantage The effect of variation of the pH with variation of environmental CO_2 was studied (Table 5)

TABLE 5

Effect of pH in Medium in Cuvettes

	Variation of pH of growth medium							
	Foetus 22 6				Foetus 17/6			
				Mitotic Index				
Time	pH				pH			
	6 6	6 9	7 2	7 6	6 6	6 9	7 2	7 6
2nd day	3/17	5/16	6 17	2/15	2 5 18	4 21	4 5/22	0 15
3rd day	4/16	7/20	4 18	1/16	4 18	6/21	4/18	0 16

RESULTS

It is clear from Table 5 that the mitotic index was highest when the *pH was 6 9–7 2*

Use of Different Sera and Inactivation of Serum

Object glasses inoculated with preparations from the same foetus were placed in cuvettes containing 20 per cent serum from persons belonging to different blood groups (Table 6)

TABLE 6

Different Sera in Cuvettes

Different sera in cuvettes	Foetus 20/4 Mitotic index	
	2 day	3 day
Bloodgroup A	5/22	4/20
Bloodgroup B	4 5 20	5 25/20
Bloodgroup AB	5/20	5 25/20
Blo dgroup O	4/21	5 25 19

RESULTS

It is clear (Table 6) that growth did not vary with bloodgroup

In one experimental serie the *addition* of inactivated (30 min at

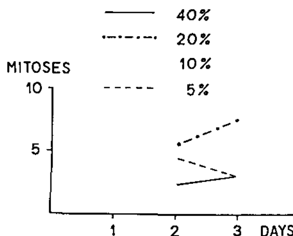
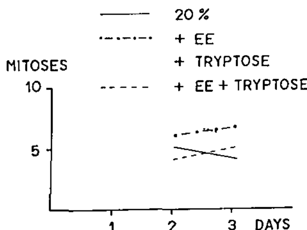


Fig 4

Effect of variation of amount of serum on number of mitoses (20 fields of vision

TABLE 4

	Mitotic index 2nd day	
20 % human serum	5 75	26 5
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Figs 5 and Table 4

Effect of addition of tryptose and embryonal extract to medium on number of mitoses (Fig 5) and on mitotic index (Table 4)

RESULTS

When serum was not added, no mitoses occurred and the cells did not appear to be healthy when they were stained 2 days later The highest number of mitoses was obtained with 20 per cent serum

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Effect of pH in Medium in Cuvettes

	Variation of pH of growth medium							
	Foetus 22 6			Mitotic Index		Foetus 17/6		
Time		pH				*pH*		
	6 6	6 9	7 2	7 5	6 6	6 9	7.2	7 6
2nd day	3/17	5/16	6 17	2/15	2 5/18	4/21	4 5/22	0 15
3rd day	4/16	7/20	4 18	1/16	4 18	6/21	4/18	0 16

RESULTS

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Object glasses inoculated with preparations from the same foetus were placed in cuvettes containing 20 per cent serum from persons belonging to different blood groups (Table 6)

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Bloodgroup B	4 5/20	5.25/20
Bloodgroup AB	5/20	5 25/20
Bloodgroup O	4/21	5 25 19

RESULTS

It is clear (Table 6) that growth did not vary with bloodgroup

In one experimental serie the addition of inactivated (30 min at

TABLF 7

Comparison between Inactivated and Fresh Serum in Cuvettes

	Mitotic index	
	uninactivated	inactivated
O serum Foetus 10/5	9/18	4 5/15
O serum Foetus 24/4	11 5/19	4 5/17

56° C) and uninactivated serum in the cuvettes was studied for any effect on growth (Table 7)

RESULTS

Inactivation of serum impairs its growth stimulating effect (Table 7) All the sera were stored at —20° C after collection Attempts were made to study the effect of storage, and the results are given in Fig 6, which compares the effect of 3 aliquots of serum prepared from one and the same blood sample, but added to the medium in fresh state (within 2 hours after puncture), after storage for a week at + 4° C and —20° C, respectively (Fig 6)

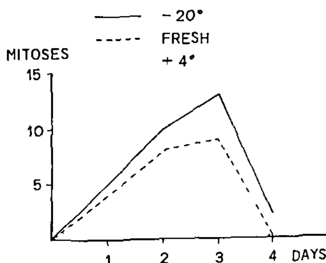


Fig 6

Effect of storage of serum on number of mitoses

RESULTS

From this experiment (Fig 6), which was repeated with other sera and with the same result, it is clear that the growth stimulating factor decreased considerably after storage of the serum for 1 week at +4° C, while storage at —20° C had no such depressive effect

Method for Testing the Effect of Small Amounts of Serum

The cuvettes could hold 23 object glasses and since it was desired to devise a method allowing the use of small amounts of serum, the latter was deposited in rings on the object glasses

In the first tests object glasses containing serum and cells added si-

f the cells

llowed to

attach during 4 hours The tests were carried out with both inactivated (56° C for 30 minutes) and uninactivated sera from healthy donors The results are given in Table 8

RESULTS

It is apparent from Table 8 that freshly prepared serum or serum stored at −50° C impairs the attachment of the cells to the object glass Inactivation of the serum by exposure to +56° C for 30 minutes has no appreciable influence on this effect On the other hand, the inactivation appeared to depress the number of mitoses, which is in accord with previous observations on addition of serum to the cuvettes In comparative tests it could be shown that storage at −20° C had no advantage

TABLE 8

Serum	Blood group		Mitotic index			
			Attached at once		After 2 hours	
			uninact	inact	uninact	inact
Ber	O	fresh	1/4 5	0 5 3	4 5/17 5	3 5/19 5
		2d + 4°	9/21	5/20	7 5 21	3 5/23 5
		2d — 50°	0/1 5	0/2 5	4/20	3/17
	O	fresh	0 1 5	0/2	7 5/20	3 5/19
		2d + 4°	6 20 5	3/18 5	9/26 5	5/21 5
	A	fresh	2 6 5	1 5/7 5	6 22 5	4 5 21
		2d + 4°	4/19 5	2 5 18 5	6 5/19	4/18 5
	B	fresh	1/9	1/8	2 5/21 5	1 5/18
		2d + 4°	3/12 5	0/15	4 5/23	2/17
Pat serum						
IX		fresh	0 5 2 5	0/1 5	4/15 5	2/12 5
X		fresh	0 3 5	0/4 5	7/17	3 5/15
XI		fresh	5 5 15 5	1 5/11	7 5/22 5	3 5/15 5
XX		fresh	3 5 11 5	0/1 5	6 5/19 5	3 5/15 5
Pooled serum from virus lab (Pooled serum from more than 20 donors) kept for more than 2 days at + 4° C						
Pool I			9/21	4/17	8 22 5	4 5/20
Pool III			6 5/16 5	3 17	6/18	5/18

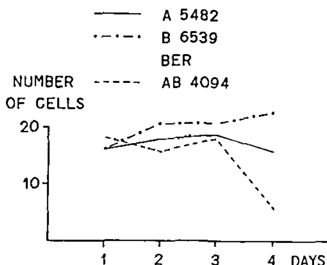


Fig 7
Serum added to cuvette

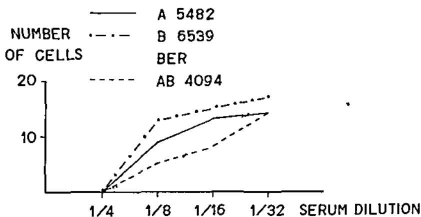


Fig 8
Serum added to ring

Figs 7 8
Comparison between the effect of the two methods on the number of cells

over storage at +4° C of the serum when cell attachment is concerned This is in contrast to the influence of storage on the growth stimulating factor

Comparative tests in which serum was added to the rings before the cell suspension and tests of the type described above showed that the sensitivity of the method particularly concerning the evaluation of the effect of the serum on the attachment of the cells to the object glass was greater when the serum was added first and the cells then allowed to sediment through the test solution Once this had been realized it was decided to determine by titration the smallest amount of serum capable of interfering with the attachment of the cells and the number of mitoses Serum in increasing dilution was deposited in the rings, to

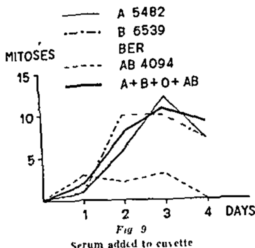


Fig 9
Serum added to cuvette

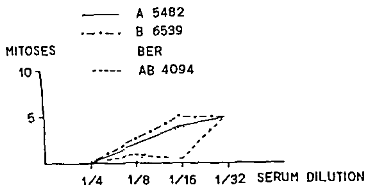


Fig 10
Serum added to ring

Figs 9 10
Comparison between the effect of the two methods on the number of mitoses

which cell suspension was then added The serum dilutions were prepared with Hank's solution and 0 2 cc was deposited in the individual rings

The results were compared with those obtained by the method described previously with test serum in the cuvette

Method for Comparison

The number of cells and mitoses were determined every 24 hours for 4 days Serum dilutions (1 4, 1 8 etc) were added in amount of 0 2 cc to the other object glasses The same cell suspension was poured over these glasses as in the above mentioned comparative experiment After the cells had been given 4 hours to attach to the glasses the latter were

rinsed in warm Hank's solution and placed in a cuvette with 20 per cent pooled human serum and EE After 48 hours the glasses were fixed and stained

The growth of cells and the number of mitoses were studied and the results are given in Figs 7, 8, 9 and 10

RESULTS

It is clear from the curves that the number of mitoses varied fairly closely with the number of cells A certain deviation from other sera was noted, particularly the number of mitoses of an AB serum Other AB sera tested did not show this tendency It is widely believed that 0 2 cc of a fresh serum diluted ¼ prevents attachment of the cells and thereby also cellular growth By diluting the serum we obtained curves for both the number of cells and mitoses The results obtained agree well with those obtained by the first used method despite the introduction of a new factor, *i e* attachment of the cells In the first method the cells were allowed to adhere in the same environments and then grow out under different conditions The new variant was devised after we had observed that fresh serum added to the rings immediately after the cell suspension influenced the adhesion of the cells and thereby the number of mitoses compared with what was seen, when the cells were first given 2 hours to attach to the glass (see Table 8)

DISCUSSION

It is clear from the foregoing presentation of the experiments and results that the method described is simple and can be readily modified to study cell growth and factors influencing such growth Ordinary object glasses are used and they are dried with a piece of linen cloth and sterilized in dry heat This avoids the otherwise time consuming cleansing of the glassware The glass plates are easy to store

Since the glasses cultured under identical conditions can be taken out of the cuvette and examined at different intervals, it is possible to follow the growth of the cells The staining methods may be varied, but, as a rule, we used haematoxylin-eosin Growth was followed by counting the number of mitoses and cells, which takes about 2 3 minutes per glass Since both the mitoses and the cells are counted, two complementary values are obtained, which together eliminate a difficulty otherwise entailed by the evaluation of injured or dead cells

The object glasses may stand in the cuvettes up to 10 days before the cells fall off In experiments with chick fibroblasts the cells were sometimes allowed to stand 20 days in the same medium (*Bergman & Jonsson* 1963)

The modified method which requires only a small amount of serum influences the attachment of the cells, but since the results obtained

agreed well with those achieved with the first used method we now generally use this modified procedure

The procedure will probably also prove useful for testing substances other than serum as well as for testing the effect of serum for example on other cells The experimental conditions such as time amount of cells types of cells and concentration of the test substances can be readily changed *ad libitum*

SUMMARY

Human embryonal fibroblasts were studied regarding variation in growth with the number of the cells in the inoculum with the donor embryo and change of nutrient medium of the primary cultures Growth was found to vary substantially with the addition of serum FF and tryptose phosphate broth to the nutrient medium The stimulating effect of the serum on the growth of the cells did not appear to vary with blood group of the donor but inactivation of the serum reduced the growth stimulating effect of the latter

A screen test for studying the effect of serum *in vitro* on human embryonal fibroblasts with the use of small amounts of serum is described

The costs of th s invest gation were defrayed l grants from the Swedish Medical Research Counc l and the Swed sh Cancer Society

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FROM THE DEPARTMENT OF HISTOLOGY OF THE KAROLINSKA INSTITUTET STOCKHOLM 60, SWEDEN

THE CENTRAL NERVOUS SYSTEM OF FOETAL MICE AFTER ADMINISTRATION OF STREPTOMYCIN

By

BIRGITTA ERICSON-STRANDVIK *and* LARS GYLLENSTEN

Received 26 iii 63

It is well known that disturbances occur in the cochlear and/or vestibular functions after the administration of streptomycin Corresponding to those physiological effects, severe histologic injuries may be found in the neuroepithelium of the inner ear It has been a point of contention whether or not these peripheral changes are complicated by central injuries affecting the nuclei of the stato acoustic nerve or other parts of the central nervous system (for references and discussion, see *Berg* 1951, *Neumann & Neubert* 1958, *Florey* 1961) However, the cerebral changes that have been reported are subtle, as far as can be judged by histological methods, and their significance remains to be decided

Using quantitative cytologic methods, *Floberg et al* (1949) were able to demonstrate inhibition of nucleic acid production in vestibular ganglion cells and in the cells of Deiter's nucleus in guinea pigs given streptomycin These findings derive support from several biochemical investigations on bacteria in which streptomycin has been found to interfere with the metabolism of ribonucleic acid (*Erdos & Ullman* 1959, *Anand et al* 1960, *Rosano et al* 1960, *Roth et al* 1960) The actual cause of such an interference remains to be elucidated It has been suggested that changes in membrane permeability play a part in this connection From other quarters, it has been contended that streptomycin produces damages to the bacterial respiratory activity (*Paine & Clark* 1954, *Umbreit* 1956, *Hahn* 1958)

A great many of the noxious influences used in experimental embryology to cause malformations are characterized by their capacity to interfere with the nucleic acid metabolism Also experimentally induced disturbances in the respiratory processes very often have teratogenic effects A general account on experimental teratology may be obtained from the J Cell Comp Physiol, Vol 43 Suppl 1 (1954), *Tondury* (1956), *Kalter & Warkany* (1959), the Ciba Foundat Sympos on Congenital Malformations (1960), and *Montagu*, Prenatal Influences (1962)

Assuming that streptomycin is in actual fact a potent general neuro toxin acting on nucleic acid metabolism and/or cellular respiration, it may conceivably be a teratogenic substance that gives rise to malformations in the central nervous system when administered to embryos during a sensitive period of neurogenesis The prerequisite for such an hypothesis is established because streptomycin penetrates the human placental barrier (*Charles* 1954)

The aim of the present investigation is to study the effect of streptomycin on neurogenesis with due consideration to the forementioned arguments The experimental animals employed were mice, as *Andre* (1959) demonstrated, using tritium labelled streptomycin that this drug passes the placenta and the blood brain barrier in these animals The sensitive periods in the embryogenesis of mice have also been clearly defined in a number of experiments with regard to x ray malformation and other experimentally induced malformations Gross neurological malformations can be produced about the middle of the mouse pregnancy (*Hicks* 1954, *Russell & Russell* 1954, *Brent* 1960)

No teratogenic action of streptomycin has been reported *Filippi & Mela* (1958) described experimentally induced malformations of the extremities in rats after the administration of streptomycin and penicillin to the mothers during pregnancy The role of streptomycin alone in these analyses cannot, however, be estimated, as it was always given together with the penicillin In reports by *Moiseeva* (1954), on the placental passage of streptomycin in rats and rabbits it was stated that a relat ... to ... ms ... was ... ...

Riskaer et al (1952) found no malformation but placental damage and increased foetal mortality in guinea pigs after the administration of streptomycin and dihydrostreptomycin The central nervous system was stated to be normal in histological examination

MATERIAL AND METHODS

C 57 Bl mice were used The female mice were isolated and vaginal smears were prepared every morning After at least *two regular* cycles the females were put together with males at oestrous over night After mating the occurrence of vaginal plug and/or gestational smear was regarded as indicative of pregnancy On days 9 13 after mating pregnant animals were given ... at 9 a m and 4 p m The drug (Strepsulfat ... muscularly 250 mg/kg of b ... the mother animals we ... in B uin s fluid After ... series The sections we ... frontally in sta ned ... ed and For a ... Strepsu ... mg of pregna ... nber of ... hours

after the injection and the streptomycin concentration was determined bacteriologically on press juice of the embryos

For histological studies 10 litters were given streptomycin comprising 58 embryos 11 litters served at controls comprising 62 embryos 29 litters were employed for records of the embryonal streptomycin concentration i e a total of 152 embryos

According to the manufacturers the LD 50 of Strepsulfat when given intravenously to white mice is 80 130 mg In our experiments the mothers given Strepsulfat exhibited signs of respiratory distress and malaise 15 30 minutes after the injections

RESULTS

The *concentration of streptomycin in the embryos* after intramuscular injection of 250 mg of streptomycin to the mothers on the 13th day of pregnancy is shown in Fig 1 Marked differences were observed *between the respective litters, being less conspicuous between the young of each litter*

No significant differences in litter size were to be found between the animals given streptomycin and the control animals The mean number of embryos in mothers that obtained streptomycin equalled 5 8 ± 0 49 and in control mothers 5 6 ± 0 46 Thus, no indications of increased embryonal mortality were to be noted after streptomycin administration

External examination of the embryos revealed *no external malformations* The brains and the cranial segments of the cervical medulla were examined microscopically *No gross malformations* were observed

In 9 embryos *slight microscopic irregularities* were ascertained

Small foci of *pycnotic cell nuclei* were observed in 4 embryos (Fig 2) Scattered pycnosis occurred in all the embryos, *i e* both in the control animals and in those given streptomycin This corresponds to the normal '*histiogenetic degeneration*' of *neuroblasts during embryogenesis* (*Hughes & Fozzard* 1961) Focal accumulations of pycnotic nuclei, however, were not seen in the control animals

In 2 embryos microscopic *haemorrhages* were noted (Fig 3) In 1 of these embryos, also a pycnotic focus occurred, as referred to above

2 embryos disclosed microscopic *perivascular accumulations of lymphocytes* and cells resembling lymphocytes (Fig 4)

1 embryo showed a *polypous excrescence* in the third ventricle (Fig 5)

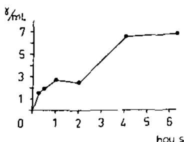


Fig 1

Concentration of streptomycin sulphate in press juice of mouse embryos 0 6 hours after a single intra muscular injection to the mother



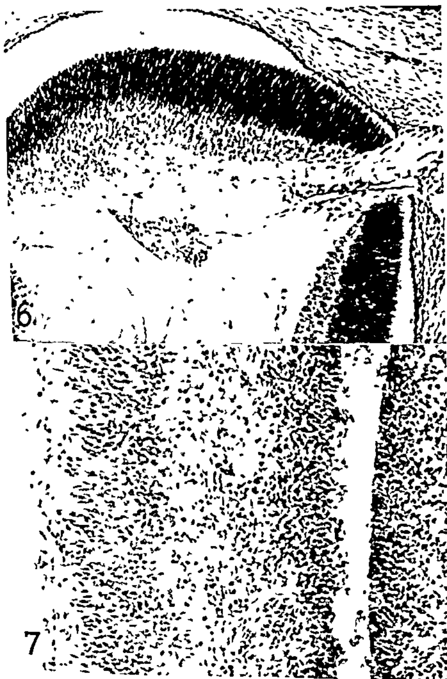
Fig 2 Group of pycnotic neuroblasts in mouse embryo after streptomycin administration to the mother The pycnotic focus is located ventrally to the fornix × 160

Fig 3 Small haemorrhage in the parietal cerebral cortex of mouse embryo after streptomycin administration × 260



Fig 4 Perivascular accumulation of lymphocyte like cells in the arachnoid of the temporal cerebral lobe in mouse embryo after streptomycin administration × 160

Fig 5 Polypous excrescence in the third ventricle of mouse embryo after streptomycin administration × 160



Figs 6 and 7
(Please turn over for the text)



Fig 4 Perivascular accumulation of lymphocyte like cells in the arachnoid of the temporal cerebral lobe in mouse embryo after streptomycin administration × 160

Fig 5 *Polypous excrescence in the third ventricle of mouse embryo after strepto mycin administration* × 160

heads prepared on the 18th day of pregnancy In 9 embryos (of a total of 52 streptomycin treated embryos) slight microscopic irregularities were seen in the brain, i e focal pycnosis, perivascular cell infiltrations, microscopic haemorrhages, ependymal polypus and eye anomaly The specificity of these findings was called in question

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In 1 embryo an *anomalous proliferation of the hyaloid vessels* was observed in one eye, the same eye manifesting also irregularities of the retina (Fig. 6). These changes are similar to those seen in growing mice after postnatal exposure to concentrated oxygen, and relating to the disease "retrolental fibroplasia" of the human being (*Gyllensten & Hellström* 1954).

None of the forementioned microscopic changes could be established in the control embryos not given streptomycin.

In addition to the microscopic irregularities recorded above, 2 animals disclosed apparent defects in the hippocampal pyramid cell layer, and several embryos showed irregularities of the ventricular ependymal linings (Fig. 7). Similar changes have been reported after roentgen irradiation of rat embryos (*Cowen & Geller* 1960, *Roizin et al.* 1962), respectively. In our series similar findings cannot be attributed to streptomycin intoxication in view of the fact that they were observed also in the control embryos in approximately the same frequency.

DISCUSSION

The significance of the microscopic findings in mouse embryos after streptomycin treatment can not be definitely evaluated. Similar changes have been reported in other studies of experimental teratology. Pycnosis, perivascular cell infiltrations and haemorrhages have, for instance, been observed after x-ray irradiation of the developing brain, and the eye anomaly has been found in growing mice with oxygen poisoning. Thus, no specific streptomycin-induced damage has been ascertainable.

Nutritional deficiency, hypoglycaemia and vitamin deficiencies may cause developmental disturbances (*Warkany et al.* 1942, 1954, 1957). Since the possibility of such or other metabolic effects of streptomycin intoxication cannot be excluded, the mechanism of streptomycin-induced irregularities of the brain eludes definition.

SUMMARY

Streptomycin sulphate was given to pregnant mice on days 9–13 of pregnancy. The drug was found to pass the placental barrier. No increase of embryonal mortality was noted. No external malformation and no gross malformation of the central nervous system were observed to judge from microscopic analysis of serially sectioned embryonal

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Figs. Cont'd

Fig. 6. Hypertrophic hyaloid vessels and retinal irregularities in the eye of mouse embryo after streptomycin administration. × 160.

Fig. 7. Ependymal irregularities in the lateral cerebral ventricle of mouse embryo after streptomycin administration. Similar findings have been reported after X-ray *treatment of mouse embryos* but are also found in our control embryos. × 160.

FROM THE UNIVERSITY INSTITUTE OF PATHOLOGY, FREDERIK DEN FEMTES VEJ 11, COPENHAGEN, CHIEF PROFESSOR GUNNAR TEILUM, M.D.

AN IMPROVED TECHNIQUE FOR HYPOPHYSECTOMY OF YOUNG MICE

By

EMMERIK JENSEN

Received 28 iii 63

The operative technique to be described is a modification of that described by *Thomas* (1938). It differs only little from that, but is in the writer's opinion easier to perform.

Under ether anaesthesia the mice are placed supine on a mouse board, the head distal to the operator and the legs fixed. It is important that the head is firmly fixed and cannot roll sideways. For this purpose, it is placed on a hollowed out wedge, the nose being lifted about 5 mm from the table level and fixed with a rubber band around the upper incisors. The operation is carried out under a dissection microscope with a magnification of × 4. A midline incision is made from the jugulum to the tip of the mandible, the salivary glands are separated by blunt dissection and kept aside by retractors. The pretracheal muscles are divided by blunt dissection a little lateral to the midline and the cleft further widened by means of a fine, curved, blunt forceps. Viewed from the operator, the trachea is now on the left, the vascular sheath with the cervical vessels on the right, and at the bottom of the field the longus capitis muscles presenting themselves as two slender muscle bands converging anteriorly. Just anterior to their insertion the sphenooccipital synchondrosis may be seen as a transverse slightly shiny line of cartilage, about 2 mm in length. In front of it the pharyngeal wall meets the base of the skull. With a scraper about 1 mm wide, the prevertebral muscles are scraped off in the antero posterior direction, laying bare the base of the skull. The pharyngeal wall is cautiously pushed forward, exposing the base slightly *in front* of the synchrondrosis. Scraping the muscles must be done with the utmost caution in order not to injure the large veins laterally on the base of the skull. With a sharp, pointed knife the occipital bone is divided about 1 *mm behind* the synchondrosis by a transverse incision, about 2 mm in length. The knife must not cut too deep, only just through the bone, as the brain stem is close to the skull at this site. Thereupon, the synchondrosis is opened, the knife splitting the cartilage as far to the anterior aspect as possible and in its full width. From the anterior incision, an approx. 2 × 1 mm flake of

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It must be pointed and sharp and its edge must not be longer than about 1 mm A suitable knife is the Gilette operation knife of the smallest size, on which the proximal part of the edge has been ground off (cf Fig 1)

If the edge is longer, it is difficult to avoid injuring the vascular sheath when cutting the base of the skull

The use of this special knife is the main difference from the technique described by Thomas, in which blunt hooks were used for opening the skull It represents in the writer's opinion a definite advantage over the use of blunt hooks, as cutting with a sharp knife with a short edge is easier to perform than breaking the base of the skull and gives less bleeding With some routine the overall mortality—both operative and later—is less than 25 per cent

A drawback of this method is that it is inapplicable for adult mice When the mice are older than about 35 days, the base of the skull has become too hard to be cut by a knife

SUMMARY

An improved technique for hypophysectomy of young mice is described

It is based on the technique of *Thomas*, but differs in the use of a special knife

REFERENCE

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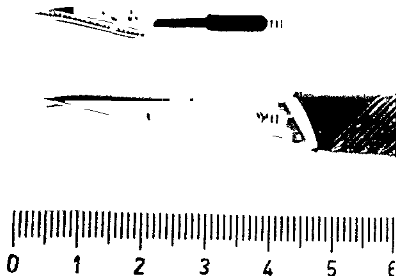


Fig 1
The knife with the ground off edge

bone may be tipped up with the knife and removed This exposes the under side of the hypophysis appearing as a ca $2 \times 1 \times 1$ mm transverse structure just above the synchondrosis It is situated in a duplication of the dura, and the parietal layer has to be split very cautiously with the knife Now, the hypophysis may be sucked out by means of a slender glass pipette with an external diameter about 1 mm, and as thin-walled as possible The suction must not be too strong, as this may *rupture the dura on the upper aspect of the hypophysis and injure the* brain stem Ordinary water suction is suitable The pipette is cautiously approached to the exposed hypophysis which is easy to suck out, as a rule in one piece therefore, the pipette must not be too small Any bleeding which might occur is absorbed by small rolls of cotton-wool, and the skin incision is closed with 2 small agraffes

When operating on young mice (*body weight 15 20 grammes*), tracheal intubation is out of the question As a result, the operation has to be carried out in several, quite short, steps allowing the mouse to breathe freely in between It is important not to compress the trachea for too long and not to scrape toofar lateral in removing the prevertebral muscles or use too strong suction, as this may entail profuse bleeding

This method permits the removal of the hypophysis under visual control and is applicable in very small animals With some practice mice as small as 10 g may be successfully hypophysectomized Little equipment is needed In addition to the microscope and mouse board, a pair of small, straight scissors, two fine blunt forceps, one straight and one angular, a slender scraper, and a knife The knife is important

MATERIAL AND METHODS

24 male guinea pigs weighing 220–310 g, were employed 12 of them were used as untreated control animals the rest being treated with thyroxin The thyroxin (Thyroxin ®, Hoffman-La Roche & Co) was given subcutaneously in the dorsum of the animals in a dose of 5 micrograms per 100 g of body weight Each treated animal received 2 such doses, the first on day *0*, and the second on day *3* On day *0* and *6* the animals were weighed and the change in body weight was registered Without any selection each thyroxin treated animal was paired with a control, and the two animals were dealt with similarly and killed on the same day

On the 6th day, when the mitochondrial increase in blood lymphocytes was maximal in accordance with previous observations (*Ernstrom & Larsson* 1961) the animals were killed by a blow on the neck Blood was taken from the internal

polynuclear cells lymphocytes and monocytes/mm³ of blood was calculated

The thymic lobes the cervical and the inguinal lymph nodes were dissected and a cell suspension of each organ was made in 0 9 per cent NaCl solution A small drop of each suspension was then transferred to a slide which had been prepared with Janus green B an

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stained mitochondria in the viable cells was counted From each suspension 200 cells were analysed

Smears of the suspensions of thymic and lymph node cells were stained with methyl green and pyronine after fixation in ethanol-chloroform acetic acid The suspensions contained mainly lymphocytes

... percentages of reticular cells

... cells included in the mitochondrial counts correspond accordingly to the lymphocytes and to the pyroninophilic cells excepting the mature plasma cells referred to in earlier papers on the thymus and lymph nodes (*Ernström & Gyllensten* 1959, *Ernström* 1963)

RESULTS

The thyroxin-treated guinea pigs as well as the normal ones increased in weight during the experimental period of 6 days The mean increase of the former was slightly less than that of the latter (+ 6 2 per cent and + 7 7 per cent, respectively) The changes of the blood picture are shown in Table 1 When the statistical analysis is ...

Figs 1 3 show that the mitochondrial contents is correlated to the cellular size Thus, large cells had a large quantity of mitochondria This correlation is apparent both in normal and in thyroxin-treated animals However, it may be inferred from columns 5 and 6 in Figs 1–3 that the large cells in the thyroxin-treated animals contain somewhat

FROM THE HISTOLOGIC DEPARTMENT OF THE KAROLINSKA INSTITUTET STOCKHOLM 60 SWEDEN HEAD PROFESSOR N A HILLARP

INFLUENCE OF THYROXIN ON THE MITOCHONDRIAL CONTENTS OF THYMIC AND LYMPH NODE CELLS

A Quantitative Study in Guinea Pigs

By

U Ernstrom and B Larsson

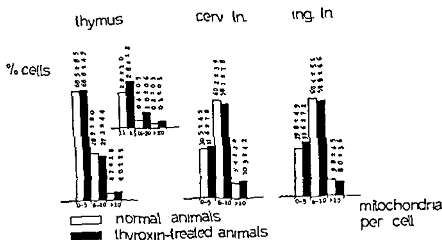
Received 13 ix 63

Exogenous thyroxin, when administered in doses adapted to avoid causing general cachexia, is known to stimulate the growth of thymo-lymphatic tissue in the young guinea pig (for references, see *Gyllensten* 1953, *Ernstrom & Gyllensten* 1959) The observations made by Japanese researchers concerning the contents of mitochondria in the blood lymphocytes during the growth of the lymphatic organs spurred the present authors to perform an analysis of the blood lymphocytes in thyroxin-treated guinea pigs (*Ernstrom & Larsson* 1961) A significant increase of the mitochondrial contents of blood lymphocytes was ascertained in the hyperthyroid animals It is the aim of the present investigation to try to shed some light on the questions that cropped up as a result of this finding

1 Is the increased frequency of blood lymphocytes rich in mitochondria, as found in the thyroxin-treated animals, the result of *a general increase of the mitochondria in cells* belonging to the thymo lymphatic system, or can it be attributed to a selectively increased delivery of cells that are particularly rich in mitochondria?

2 Is there any difference between thymic cells and lymph node cells as regards their contents of mitochondria? *Do the mitochondrial* contents change during thyroxin treatment? If so, can such a change give a hint of the source of the blood cells rich in mitochondria in the thyroxin treated animals?

3 Are the mitochondrial contents of the thymic and lymph node cells correlated to the size of the lymphocytes? Do the *blood lymphocytes* rich in mitochondria reflect processes of growth in the lymphatic tissue (*Imamura* 1959) and the thymus?



Figs 4–6

Percentage distribution of cells with different mitochondrial contents in thymus, cervical and inguinal lymph nodes of normal and thyroxin treated guinea pigs

TABLE 2

Mean Number of Mitochondria in Thymic and Lymph Node Cells in Normal and Thyroxin Treated Guinea Pigs (Mean ± Standard Deviation)

	Number of animals	Thymus	Cervical lymph nodes	Inguinal lymph nodes
Controls	12	4 8 ± 0 4	7 0 ± 0 5	7 0 ± 0 5
Thyroxin treated	12	4 9 ± 0 3	7 1 ± 0 5	6 8 ± 0 4

without a corresponding increase of the mitochondrial contents, or to an intensified emigration of the large cells that have the largest contents of mitochondria

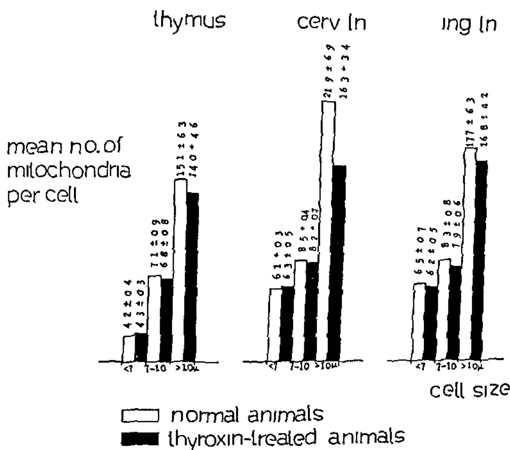
Figs 4–6 demonstrate the distribution of cells with different mitochondrial contents in the thymus, cervical and inguinal lymph nodes As regards the contents of mitochondria, the cell populations of the lymph nodes differ clearly from those of the thymus In the latter the majority of the cells (69 per cent) belongs to the first mitochondrial class (0 5 mitochondria) and in the cervical and inguinal lymph nodes the majority (60 per cent) belongs to the second mitochondrial class (6–10 mitochondria) The differences are evident also from the mean mitochondrial number per cell (Table 2)

The mean number of mitochondria per cell in the thymus and in the lymph nodes was not changed after the thyroxin-treatment (Table 2) When the distribution of the cells in the normal and thyroxin-treated animals is compared, the thymus of the latter group reveals a greater frequency of cells rich in mitochondria Statistical analysis proved that the difference of the means was almost significant ($p < 0\ 05$) In Fig 4

TABLE 1

Changes of Blood Picture in Thyroxin-Treated and Normal Guinea Pigs
T = Thyroxin treated C = Controls

	Mean $\pm s_d$	t	df	p
Total white blood cells/mm³	T 3880 ± 1340 C 2905 ± 1280	1 8	22	< 0 1
Polynuclear cells/mm³	T 883 ± 683 C 743 ± 676	0 5	22	< 0 7
Lymphocytes/mm³	T 2339 ± 1116 C 1740 ± 1089	1 3	22	< 0 2
Monocytes/mm³	T 656 ± 429 C 420 ± 256	1 6	22	< 0 2



Figs 1-3

Correlation between number of mitochondria per cell and cell size in thymus cervical and inguinal lymph nodes of normal and thyroxin treated guinea pigs

less mitochondria than those in the untreated animals These differences were not statistically significant, but the trend is the same in all three organs under examination This tendency, if not merely a chance phenomenon, may either be due to a production of large cells

node cells, was increased during the postnatal growth Further, he considered the possibility of a correlation between hyperplasia of the thymus and the mean number of mitochondria in the blood lymphocytes These observations seem comparable to the changes now presented in the mitochondrial contents of thymic cells and blood lymphocytes, seen during a thyroxin-induced proliferation of cells in the thymus and lymph nodes

Studies of the number of mitochondria in the blood lymphocytes during growth processes in the thymo-lymphatic system have been performed mainly by Japanese authors (for references, see *Ernstrom & Larsson* 1961) In accordance with *Wiseman* (1931) and more recently *Fichtelius & S E Larsson* (1961), they assumed these lymphocytes rich in mitochondria to be young cells with a capacity to differentiate into small lymphocytes or other cell types, *i e* plasma cells *Wiseman's* (1931) contention that large lymphocytes contain more mitochondria than small ones, which was supported by *Fichtelius & S F Larsson* (1961), is confirmed by the present findings and can be applied to the cell populations of the thymus and lymph nodes

It seems reasonable to conclude, on the basis of the studies of the influence of thyroxin on the thymo-lymphatic tissues, that the hormone stimulates a proliferation of pyroninophilic cells rich in mitochondria in the thymic cortex and the lymph node medulla, and increases the delivery of these cells into the blood especially from the thymus The destination of these lymphocytes is still unknown, but observations made by *Fichtelius* (1953, 1958) would indicate that these cells will to a large extent settle down in the spleen

SUMMARY

(1) Cell suspensions derived from the thymus, the cervical and inguinal lymph nodes of normal and thyroxin treated guinea pigs were examined by means of mitochondrial counts in living cells stained supravitally with Janus green B and neutral red The cells in the suspensions were also examined after staining with methyl green and pyronine

(2) Thyroxin does not induce a general increase of the mitochondrial contents of cells in the thymus and lymph nodes of guinea pigs The thyroxin induced increase of the mitochondrial contents of blood lymphocytes, ascertained earlier, is most probably due to a selectively increased delivery of cells rich in mitochondria to the blood

(3) In normal as well as in thyroxin-treated [illegible]
node [illegible]
mic c[illegible]
the th[illegible] [illegible]quency of cells rich in mitochondria was

(inset) the distribution of the thymic cells with more than 10 mitochondria, subdivided into 3 mitochondrial classes is illustrated In each of these three classes, the percentage of cells rich in mitochondria in the thyroxin-treated guinea pigs exceeded that of the control animals

DISCUSSION

From an extensive literature on the blood picture in human thyrotoxicosis (reviewed by *McCullagh & Dunlap* 1932, *Hertz & Lerman* 1932, *Bistrom* 1946) it emerges that thyroid hyperactivity is associated with lymphocytosis and monocytosis A similar tendency was noted in the hyperthyroid guinea pigs studied in the present investigation (Table 1) However, the latter animals were only slightly thyrotoxic, as may be seen from the almost normal increase of the body weight It is conceivable that this added quantity of mononuclear blood cells is correlated with the occurrence of blood cells rich in mitochondria, as observed in the thyroxin-treated guinea pigs (*Ernstrom & Larsson* 1961)

It is demonstrated in the present work that thyroxin, in the doses employed, does not induce a general rise in the mitochondrial contents of cells in the thymus and lymphatic tissue Therefore, the increase of the mitochondrial contents of blood lymphocytes in guinea pigs, given thyroxin in the same doses, may in all likelihood be attributed to an accentuated output of selected cells that are characterized by high mitochondrial contents

In the thymus and in the lymph nodes, the only change observed in the distribution of the cells with regard to their contents of mitochondria referred to the thymus That organ contained more cells rich in mitochondria in the hyperthyroid animals than in the controls By means of differential counts it has been possible to establish that thyroxin stimulates the proliferation of pyroninophilic cells in the thymic cortex and probably also the delivery of cells from the thymus (*Ernstrom* 1963) It is most likely that this increase of pyroninophilic cells corresponds to that of cells rich in mitochondria It is, moreover, conceivable that these cells contribute to the increased number of blood lymphocytes rich in mitochondria seen in hyperthyroid animals (*Ernstrom & Larsson* 1961) However, in normal as well as in thyroxin treated animals, the frequency of cells rich in mitochondria predominated in the lymph nodes as compared to the thymus Accordingly, even though the thyroxin-treatment did not result in any change of the distribution of mitochondria rich cells in the lymph nodes, the lymphatic tissue should not be eliminated as a possible source of the blood lymphocytes, rich in mitochondria, found in the thyroxin-treated animals

Earlier quantitative studies have been performed by *Otani* (1957) of mitochondria in tissue lymphocytes He demonstrated that in the rabbit the mitochondrial contents of thymic lymphocytes, but not of lymph

node cells, was increased during the postnatal growth Further, he considered the possibility of a correlation between hyperplasia of the thymus and the mean number of mitochondria in the blood lymphocytes These observations seem comparable to the changes now presented in the mitochondrial contents of thymic cells and blood lymphocytes, seen during a thyroxin induced proliferation of cells in the thymus and lymph nodes

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(inset) the distribution of the thymic cells with more than 10 mitochondria, subdivided into 3 mitochondrial classes is illustrated. In each of these three classes, the percentage of cells rich in mitochondria in the thyroxin-treated guinea pigs exceeded that of the control animals.

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In the thymus and in the lymph nodes, the only change observed in the distribution of the cells with regard to their contents of mitochondria referred to the thymus. That organ contained more cells rich in mitochondria in the hyperthyroid animals than in the controls. By means of differential counts it has been possible to establish that thyroxin stimulates the proliferation of pyroninophilic cells in the thymic cortex and probably also the delivery of cells from the thymus (*Ernstrom* 1963). It is most likely that this increase of pyroninophilic cells corresponds to that of cells rich in mitochondria. It is, moreover, conceivable that these cells contribute to the increased number of blood lymphocytes rich in mitochondria seen in hyperthyroid animals (*Ernstrom & Larsson* 1961). However, in normal as well as in thyroxin-treated animals, the frequency of cells rich in mitochondria predominated in the lymph nodes as compared to the thymus. Accordingly, even though the thyroxin treatment did not result in any change of the distribution of mitochondria-rich cells in the lymph nodes, the lymphatic tissue should not be eliminated as a possible source of the blood lymphocytes, rich in mitochondria found in the thyroxin-treated animals.

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increased These cells may contribute to the blood lymphocytes rich in mitochondria seen in hyperthyroid guinea pigs.

(4) The contents of mitochondria in the thymic and lymph node cells were correlated to the cellular size. The cells rich in mitochondria were large and pyroninophilic. It would appear plausible that the mean mitochondrial contents of the blood lymphocytes reflect processes of growth in the thymo-lymphatic tissue.

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FROM THE DEPARTMENT OF PATHOLOGY, SECTION II,
UNIVERSITY OF HELSINKI

STUDIES ON THE ELIMINATION OF GRANULOCYTES IN THE INTESTINAL TRACT IN RAT

By

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and KYLLIKKI KIVINIEMI

Received 16 iv 63

It is an established fact that the daily production of granulocytes is enormous (8) In a state of stability the elimination must equal the production and, at least after transient disturbances, even regulate it However, our knowledge of the physiological removal of granulocytes from the animal organism is deficient One reason for this may be the numerous difficulties and ambiguities associated with the use of classical and even modern methods of investigation and the interpretation of the results achieved

"Unusual" changes have been observed in the granulocyte counts in blood on resecting parts of the ileum in rats (24) It was assumed earlier by *Pitzurra & Frascarelli* (18) that the elimination of granulocytes takes place in gastrointestinal canal Since few recent investigators have suggested the possible role of this route in the physiological elimination of leukocytes (2, 3), we decided to attempt a direct study of the elimination of granulocytes in the intestinal tract in rat

MATERIAL AND METHODS

Adult male rats of Sprague Dawley strain c 250 g in weight were used as test animals Because of the numerous difficulties involved *in the* use of accurate methods of investigation of the present problem several techniques were chosen for sampling and analysis

Sampling

Histology Samples were taken for histological *preparations* from the stomach jejunum ileum caecum and colon of 10 rats after decapitation The pieces of tissue were placed immediately in 10 *per cent neutral* formalin for 24 hours for fixing After the usual procedure and embedding in paraffin they were cross sectioned at about 5 μ around the middle of the *sample* Unfixed samples taken from two additional rats were sectioned by cryostate microtome and used for histo chemical studies

Sponsored by the *Damon Runyon Memorial Fund* (DRG 644) New York, and by the Sigrid Jusélius Foundation Helsinki

Touch samples

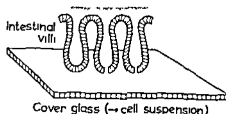


Fig 1

Contact method for sampling of granulocytes from the intestinal mucosa

Contact method (touch samples) The intestine of 7 rats was incised contra mesenterially in the longitudinal direction under ether anaesthesia the contents removed carefully and recovered from a measured lenght of the intestine The surface of the mucosa was touched lightly with a cover glass (Fig 1) treated with albumin glycerin and the cells adhering to the glass were suspended immediately in 0 1 M phosphate buffer (pH 7 0) or, after air drying stained on glass Touch samples were taken from different parts of both the ileum and the colon using contact periods of different length After this procedure some of the animals were deca pitated and histological samples were taken from the contact points to establish possible mucosal trauma

Intestinal contents The contents recovered were suspended immediately in a given volume of 0 1 M phosphate buffer (pH 7 0) After a few minutes, when the pre cipitate had settled to the bottom of the test tube, the peroxidase activity of the supernatant was determined and its cells counted in the counting chamber A few smear preparations of the supernatant and squash preparations of small content samples were also made After air drying they were stained on glass

Isolated loop of small intestine An operation was performed under ether anaesthe sia on 4 rats in which a 4-12 cm lenght of the small intestine was isolated taking good care not to injure the mesenterial veins A polyethylene catheter (Ø 1 mm) was left inside the intestinal loop and directed as shown in Fig 2 via the abdominal coverings The intestinal loop was carefully rinsed an average of once in 24 hours with 0 1 M phophate buffer (pH 7 0), using a tuberculin syringe The cells in the irrigation fluid were counted in the counting chamber smear preparations were studied and the peroxidase activity was determined after homogenization

Isolated loop of intestine

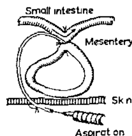


Fig 2

Sampling of granulocytes from isolated loop of small intestine

Identification and Counting of Granulocytes

Ordinary histological sections were stained with haematoxylin eosin and by a benzidine technique which stains only eosinophils (20) Frozen sections were stained by a peroxidase technique described earlier which is specific for all granulo cytes (21)

The cells in the suspension liquids were counted in Fuchs Rosenthal's counting chamber by staining the granulocytes by the peroxidase technique (21) and the eosinophils with phloxine in propylene glycol. The cell counts were generally made blind by 2 persons from 4–5 parallel samples. After rejection of the extreme values the means were calculated and entered as the final cell counts.

The smears and both the touch and the squash preparations were stained after air drying by the peroxidase technique (21) or with May Grunwald-Giemsa.

In order to compare the measured quantities of granulocytes all counts are expressed as numbers of cells per unit lenght of intestine measured in the connection of sampling.

Biochemical Methods

According to previous observations (21 23) biochemically determined peroxidase activity in different tissues in rat seems to be a measure of the total granulocyte count. Since actual results have been consistent with this assumption in all organs studied thus far (22) an attempt was made to apply the principle in the present study.

The peroxidase activity of the cell suspensions was determined spectrophotometrically from 4–5 homogenized parallel samples. The guaiacol method (7) with 2,4-dichlorphenol added as the specific catalase inhibitor (10) was used initially for the determinations. It appeared during the investigation that the guaiacol method was not serviceable in the present study. Its greatest drawback was an interfering factor(s) contained in the samples which changed the enzymatically oxidized guaiacol into a colourless compound. The ratio between this factor and the peroxidase activity varied so much in samples from different sites and from different animals that it was not possible adequately to standardize the method. In the later phase of the work consequently a change was made to the uric acid method recommended by Agner for the determination of myeloperoxidase activity 1). Th s meth [illegible]

[illegible] granulocyte amounts corresponding to the peroxidase act [illegible]

RESULTS

Light microscopy. Profuse granulocytes were established in all the histological specimens examined (stomach, jejunum, ileum, caecum, colon) (Fig 3). They were localized chiefly in the lamina *propria*. However, in the small intestine, especially in the ileum, granulocytes were also demonstrable between the epithelial cells (Figs 4, 5 and 6). The majority of the granulocytes of the intestinal wall were fairly intact morphologically but the number of disintegration forms (Fig 7) began to increase on approaching the tips of the villi. The phenomenon was especially clear in the small intestine. Granulocytes were readily demonstrable also in the intestinal lumen in the histological preparations. Most of these were typical disintegration forms of granulocytes.

The majority of the granulocytes in the *intestinal tract* [illegible] re identi[illegible] [illegible] identify [illegible] (Fig 5) [illegible] the present study to count the neutrophils in the histological sections.

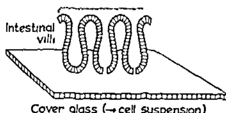


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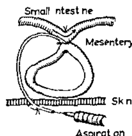


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Fig 4

Numerous leukocytes between the epithelial cells in ileum villus Most of the cells resemble lymphocytes but some of them are probably granulocytes The ultra structure of these cells as seen in electron microscopy seems not contradict this suggestion Note also the detachment of epithelial cells at the villus tip and the morphological appearance of the defect suggesting that this may well occur in physiological conditions Haematoxylin eosin staining magnification 600 ×

Fig 3

General view of the magnitude of granulocyte amounts in rat small intestine
Note that the granulocytes are the only stained cells in the picture
Frozen section peroxidase reaction

a) Magnification 80 ×
b) Magnification 320 ×, detail of the same field as in a)

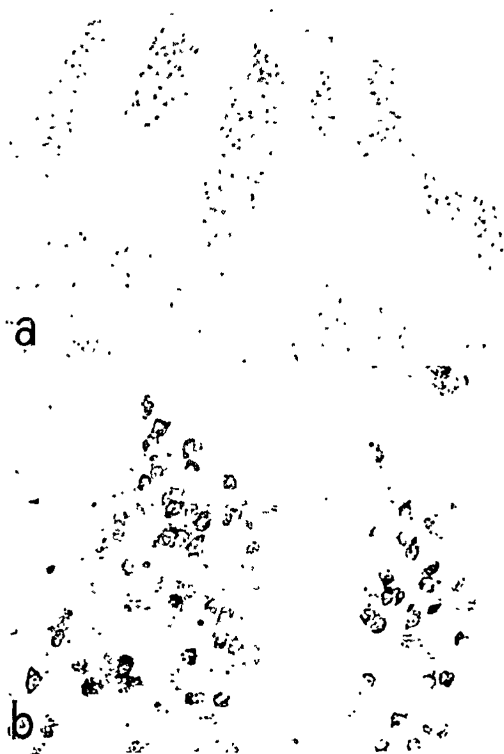


Fig 3



Fig 6

Granulocytes between epithelial cells and in *lamina propria* in ileum villus All the stained cells i e cells with dark cytoplasm are peroxidase positive granulocytes Frozen section peroxidase reaction phase contrast *microscopy*

a) Note the unusually large conglomerate of granulocytes near the edge of the left villus Magnification 240 ×

b) Note the spindle shaped granulocyte between the epithelial cells Magnification 240 ×

microscopy of living intestinal mucosa in rabbit that transient epithelial defects (gaps) of 1 to 5 cells were produced when different nutritive solutions were dropped on the mucosa Despite of these direct observations however Ritter was unable to establish any signs of the gaps in histological preparations obtained postmortally (19) The removal at the villi tips of epithelial cells that have completed their life and the extremely rapid turnover time (c 1 5 days) of the epithelium of the small intestine (14) also point to the existence of epithelial defects



Fig. 5
Leukocytes between epithelial cells in ileum villus. The cell just above the basement membrane is probably a neutrophil. Haematoxylin eosin staining, magnification 1200 ×

In agreement with earlier observations (14), a typical extrusion zone formed by destructed epithelial cells was often demonstrable at the tips of the villi of the small intestine. It was obviously in consequence of the detachment of such a mass of epithelial cells that epithelial defects of different size were also demonstrated at the tip of the villi in the small intestine (Fig. 4), but usually not in the stomach and the large intestine. These defects sometimes displayed both granulocytes and bacteria.

The defects were probably not always artefacts. This was suggested by their commonness in the ileum and scarcity elsewhere and by their morphological appearance (Fig. 4). The artefact possibility is also contradicted by the entry into the defects of granulocytes and bacteria from opposite directions. On the other hand, certain other observations as well make it very probable that such epithelial defects occur in physiological conditions. It was observed by *Ritter* (19) in direct

TABLE 1

Elimination of Granulocytes in Small and Large Intestine in Male Rat

Method of sampling	Number of granulocytes per cm of intestine (counts in chamber)		Number of eosinophils per cm of intestine (counts in chamber)	Number of granulocytes per cm of intestine calculated from peroxidase activity*
	Average	Ranges	Average	
Contact method'				
Small intestine	50×10^3	$25\text{–}100 \times 10^3$	15×10^3	200×10^3
Large intestine	100×10^3	$50\text{–}150 \times 10^3$	30×10^3	200×10^3
Intestinal contents				
Small intestine	30×10^3	$10\text{–}50 \times 10^3$	6×10^3	200×10^3
Large intestine	80×10^3	$40\text{–}150 \times 10^3$	16×10^3	200×10^3
Isolated loop of small intestine §	$1\ 2 \times 10^6$	$0\ 6\text{–}3\ 3 \times 10^6$	$0\ 36 \times 10^6$	$1\ 5 \times 10^6$ †

' The al [illegible]

§ [illegible]

† [illegible] rat [illegible] cm of intestine For another 3 rats the guaiacol method was used which, although the values are not presented here, showed strong activities (see pp 313 and 321)

cover glass from the mucosa of the small intestine was c 70 per cent The majority of both cell types were disintegration forms (Fig 9)

Of the total cell population adhering to the cover glass, c 60 per cent were other than granulocytes The majority of these cells consisted of mononuclear and epithelial cells It was not possible to demonstrate erythrocytes in the touch samples Because the ability of cells to adhere to the cover glass is probably not the same for all cell types, the proportions given are approximate

No signs of mucosal trauma caused by the contact method were observed in the histological preparations

Intestinal contents Identifiable granulocytes, *i e* peroxidase-positive cells (21), in the contents of both small and large intestine were *slightly fewer* than in the touch samples per cm of intestine (Table 1) There [illegible] in the granulocyte amounts [illegible] e smear and squash prepara- [illegible] testinal contents were breakdown forms

Isolated loop of small intestine The total number of granulocytes kept fairly evenly at 1 2 million per cm of intestinal loop in the irrigation samples taken once per 24 hours (Table 1) The relative proportion of granulocytes in the total cell population was approximately the same as in the touch samples, but there was a distinct difference in the ratio between neutrophils and eosinophils The proportion of neutrophils was at its maximum and that of eosinophils was at its minimum



Fig 7
Disintegrating granulocytes in intestinal villus Frozen section peroxidase reaction

The fairly frequent detachment of a violent character of the epithelial cell mass and the origination of the defects could thus be readily understood even from the mechanical trauma caused by intestinal contents and from the active movements of the villi in which each villus contracts to roughly a half its original length an average of 6 times per minute (13, 16) In recent studies of resorption of certain substances from rabbit intestine observations have been made suggesting that it occurs through gaps of some kind (17)

Contact method The number of granulocytes that adhered in a single touch of the cover glass against the mucosa of the small intestine was c 50×10^3 per cm of intestine (Table 1, Figs 8 and 9) An average of 100×10^3 granulocytes per cm of intestine adhered to the glass from the large intestine, the diameter of which was roughly double that of the small intestine Because of the approximation of the cell counts the ratio of the granulocyte amounts in the small and the large intestine is probably not quite correct In fact, according to the biochemical peroxidase activity determinations, no notable difference in the number of granulocytes per cm of intestine was observed The activities established by the uric acid method corresponded to higher granulocyte values than were given by the counting chamber (Table 1)

The number of granulocytes adhering to the cover glass when a prolonged contact period was used is seen in Fig 10

The proportion of neutrophils in the granulocytes adhering to the

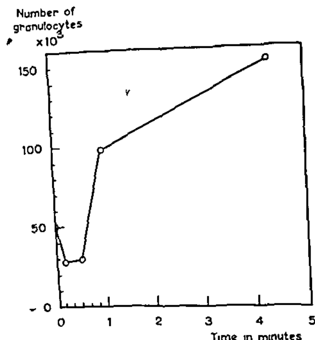


Fig 10

Number of granulocytes per cm of small intestine adhering to the cover glass in touch samples when prolonged contact periods were used

on the 2nd and 3rd postoperative day This was obviously due to local irritation caused by the operation and to a general stress leading to transient eosinopenia

The irrigation samples were often faintly greenish in colour, suggestive of myeloperoxidase liberated from the destructed granulocytes It was in fact established in the biochemical activity determinations that centrifuged cell free supernatant possessed distinct peroxidase activity and that the activity of the sample as a whole was higher than that of the touch and content specimens containing a comparable number of granulocytes

Erythrocytes were demonstrated in only one irrigation sample, taken on the first postoperative day Nor were haemoglobin absorption bands seen in any other irrigation samples when the hand spectroscope was used

Quantity of mucosal diapedesis Table 1 gives the numbers of granulocytes per cm of intestine eliminated in the small and large intestine of rat according to different methods Exact calculation of the total removal in 24 hours from these values must be based on several assumptions which were not studied in the present work Thus no details of these calculations will be presented It can be estimated however,



Fig. 8

Numerous granulocytes adhered to the cover glass touched lightly against ileum mucosa. Peroxidase reaction and counterstaining of the nuclei with haematoxylin. Magnification 200 ×

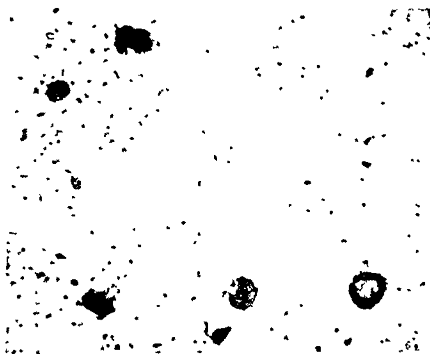


Fig. 9

Disintegrated granulocytes on cover glass obtained by the contact method. After peroxidase reaction, stained with nitroprusside. Magnification 450 ×

wall rather than arguing against the possible rôle of this route in the physiological elimination of neutrophils makes it an expected finding

Since the identification of neutrophils in tissue samples is often very difficult, unequivocal statements about the quantities of these cells in the intestinal tract are more or less invalid On the other hand, however, there is reason to believe that the scarcity of neutrophils seen in histological sections of the intestinal wall is more ostensible than real Among the arguments which seem to justify this point of view are the morphological changes which take place when the granulocytes are disintegrated (4) and even when they perform their established function (12) Regarding the last-mentioned fact, it may be mentioned that an extremely rapid degranulation of neutrophils during phagocytosis has been observed in phase contrast cinematography (12) Hence it seems apparent that changes in the characteristic features of neutrophils may lead to serious underestimations in quantitative studies

From the results of the present work and according to the studies cited it seems justifiable to conclude that the gastrointestinal tract constitutes the principal site of granulocyte elimination in a normal state of equilibrium in rat This is supported by a teleological point of view even in normal conditions the gastrointestinal canal should be considered the major potential route for an invasion of bacteria and other toxic agents, hence an effective defense system is essential in this area In fact, the importance of a defense barrier formed by granulocytes has been supported by recent observations (6) It is well known that bacterial invasion from the intestine is manifested after whole body irradiation at a time when granulocytopenia occurs However, the number of granulocytes is not decreased in blood and bone marrow only, but also very seriously in the gastrointestinal tract The epithelial damage in the intestine, on the other hand, has already been repaired by then (6)

SUMMARY

The elimination of granulocytes in the intestinal tract of adult male rat was studied In addition to the customary morphological methods, use was made of elimination of methods especially evolved for the purpose the contact method (touch samples) and isolated intestinal loop Granulocytes were counted from the samples obtained by these means and from the intestinal contents both directly in a counting chamber after peroxidase staining and also using spectrophotometrically determined peroxidase activity

It was possible to demonstrate by all the methods employed a pronounced elimination of granulocytes in the intestinal tract The gastrointestinal tract of rat seems to constitute the principal site of granulocyte elimination in a normal state of equilibrium

that a total elimination of 100×10^6 granulocytes per day and per 100 g of body weight in the intestinal tract is not implausible.

From the values given by *Donohue et al.* (9) for the total number of granulocytes in rat bone marrow and from the present knowledge of granulocyte kinetics (8) the average number of granulocytes produced in 24 hours should be in the neighborhood of $100–150 \times 10^6$ per 100 gm of body weight. Thus the estimated relationship between the production and the elimination via rat intestine shows that the gastrointestinal tract seems to be the principal site of granulocyte elimination.

DISCUSSION

The literature includes several reports of granulocytes in the gastrointestinal wall, often observed between the epithelial cells and sometimes also in the lumen (11, 15, 25). More recent reports (2, 3, 5), on the other hand, pay but little attention to the rôle of the intestinal tract in the physiological removal of leukocytes, especially in the quantitative sense. *Pitzurra & Frascarelli* (18) studied the total cell counts of the urine, saliva, gastric secreta, bile, and faeces of 10 men and then, from differential cell counts, calculated the total number of leukocytes removed via the gastrointestinal canal in 24 hours. According to them the intestinal canal constitutes the most important site of leukocyte elimination. More recently, very few workers have referred to the possible rôle of the gastrointestinal tract in the removal of leukocytes, and even they do not cite any quantitative values. *Ambrus & Ambrus* (2), however, observed in dog that labelled lymphocytes were eliminated mainly via mucosal diapedesis.

Our aim in the present investigation was to show by different methods the significance of the gastrointestinal tract in the elimination of granulocytes in normal conditions in rat. The results indicate that the intestinal tract, especially the small intestine, seems to function as principal site of granulocyte elimination in rat.

It was observed in the present investigation that the proportion of neutrophils in the granulocytes adhering to the cover glass from the mucosa of small intestine was c. 70 per cent. The observations from the histological sections, however, seem to show that the majority of the identifiable granulocytes in intestine are eosinophils. Though this paradoxical situation could be explained by a number of factors, it is our opinion that the most reasonable explanations are based on following facts.

In a state of stability the total number of different granulocyte types in various compartments must be directly proportional to the turnover times in these compartments. The available data in the literature, as well as observations in our laboratory, suggest that the time eosinophils remain in the tissues is definitely longer than that of neurophils (20). It follows that the numerical domination of eosinophils in the intestinal

wall rather than arguing against the possible role of this route in the physiological elimination of neutrophils makes it an expected finding.

Since the identification of neutrophils in tissue samples is often very difficult unequivocal statements about the quantities of these cells in the intestinal tract are more or less invalid On the other hand however, there is reason to believe that the scarcity of neutrophils seen in histological sections of the intestinal wall is more ostensible than real Among the arguments which seem to justify this point of view are the morphological changes which take place when the granulocytes are disintegrated (4) and even when they perform their established function (12) Regarding the last mentioned fact it may be mentioned that an extremely rapid degranulation of neutrophils during phagocytosis has been observed in phase contrast cinematography (12) Hence it seems apparent that changes in the characteristic features of neutrophils may lead to serious underestimations in quantitative studies

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THE EFFECT OF THYROXINE ON WOUND HEALING IN NORMAL AND ASCORBIC ACID DEFICIENT GUINEA PIGS

By

OLE JORGENSEN

Received 16 ix 63

It is generally accepted that ascorbic acid is an essential factor in wound healing in man and in the guinea pig. Very little is known, however, as to the exact mode of action of this vitamin on granulation tissue.

Robertson & Hinds (24) and *Slack* (27) have shown that the concentration of hyaluronic acid is increased in carrageenin provoked granulomas of scorbutic guinea pigs. In accordance with this finding *Jorgensen* (16) found the relative water content and the hexosamine concentration in granulation tissue from open wounds to be increased in ascorbic acid deficient animals. As the thyroid is known to exert a profound influence on connective tissue in general and seems to play a rôle in the regulation of the concentration of hyaluronic acid in this tissue (see e.g. *Asboe Hansen* (1)) it has been considered of interest to investigate the effect of thyroxine administration on granulation tissue formation in normal and ascorbic acid deficient animals.

MATERIAL AND METHODS

Female guinea pigs weighing about 300 g and female rats weighing 100 *g* were used.

[illegible]

This investigation was aided by a grant from the *Danish Leage against Rheumatism*.

The author is grateful to dr. J. *Faber* M.D. the Danish State Serum Institute for gifts of preparations of streptococcal hyaluronidase and to *Glaxo Ltd.* for a gift of thyroxine.

Miss *Lene Clausen* and Mrs. *Dorrit Lützhøft* are thanked for excellent technical assistance.

Treatment with Thyroxine

Guinea pigs 50 microgrammes of sodium levo thyroxine were injected daily suspended in 0.5 ml of physiological saline

Rats 50 microgrammes of sodium-levo thyroxine were injected daily suspended in 0.5 ml of physiological saline

All injections were given intraperitoneally. The control animals received corresponding amounts of physiological saline. In all cases treatment with thyroxine was started 5 days before surgical intervention.

Formation of granulation tissue The technique described by *Rudas* (25) was employed. With the animal under ether anaesthesia a circular piece of skin was excised medially on the back. A plastic ring was inserted in the resultant hole. The diameter of the ring was so much larger than the hole that the ring was kept fixed. All the rings had exactly the same inner diameter (20 mm) so that quantitative measurements of the granulation tissue formed were possible. The animals were killed 8 days later and the granulation tissue removed.

The amount of wet and dry, defatted granulation tissue as well as the hydroxyproline and hexosamine concentrations were determined as described by *Jorgensen & Schmidt* (14). The hydroxyproline multiplied by the factor 7.46 is a measure of the collagen content (*Neuman & Logan* (21)).

Granulation tissue removed for histological examination was fixed in 4 per cent formaldehyde solution for 4 hours. This fixation according to *Curran* (7) and *Christensen* (6) does not destroy metachromasia due to acid mucopolysaccharides.

The sections were imbedded in paraffin, cut in sections at 5 my, and stained with haematoxylin eosin, v. Gieson Hansen's stain for collagen, PAS, alcian blue, Hale, azur A. For details see *Jorgensen* (15).

Enzyme incubations Sections to be stained with PAS, Hale and alcian blue were in some instances incubated with testicular hyaluronidase (Penetrase®) or streptococcal hyaluronidase. The sample of streptococcal hyaluronidase was kindly placed at the author's disposal by dr. V. *Faber* of the Danish State Serum Institute, Copenhagen (10). This enzyme is specific to hyaluronic acid (3).

Before staining with azur A some of the preparations were incubated with pepsin.

For further details of the incubations with the enzymes see *Jorgensen* (15) and *Pearse* (22).

RESULTS

Table 1 shows the influence of administration of thyroxine on granulation tissue of guinea pigs. In normal animals treatment with this compound leads to a considerable reduction of the amount of wet and dry, defatted granulation tissue, whereas the relative water content, expressed as g water per 100 g dry, defatted granulation tissue is not influenced by this treatment. No alterations of the concentrations of hexosamine and collagen are found due to the treatment given. The amount of wet and dry, defatted granulation tissue is greatly increased in animals deficient of ascorbic acid. The relative water content and the hexosamine concentration too are increased, whereas the concentration of collagen is considerably reduced. When these animals are treated with thyroxine both the amounts of wet and dry, defatted granulation tissue are reduced, as is the relative water content. Treatment with thyroxine does not result in significant changes of the concentrations of hexosamine or hydroxyproline.

Table 2 shows the influence of thyroxine on granulation tissue formation in the rat. The numerical values of both wet and dry, defatted granulation tissue as well as the concentration of collagen are elevated by the treatment given, but none of the changes found are significantly

TABLE 1

The Influence of Thyroxine on Granulation Tissue of Normal and Ascorbic Acid Deficient Guinea Pigs

	n	Weight of wet tissue	Weight of dry defatted tissue	Relative water content	Hexosamine	Hydroxy proline	Collagen
Control	18	654 (20)	107 (7)	507 (14)	830 (18)	2332	17 40 (0 38)
Thyroxine treated	17	447 (19)*	81 (3)*	485 (7)	835 (31)	2339	17 45 (0 35)
Ascorbic acid deficient	16	1340 (82)	195 (10)	568 (11)	1009 (35)	559	4 17 (0 31)
Ascorbic Acid deficient + thyroxine	16	943 (46)*	151 (6)*	510 (17)§	914 (28)	600	4 48 (0 24)

* Significantly different from control P < 0 001 § Significantly different from control P < 0 01

The weights of the wet and dry, defatted granulation tissue are given in mg the relative water content is expressed as g water per 100 g dry defatted granulation tissue whereas the hydroxyproline, hexosamine, and collagen concentrations are given as mg mg and g per 100 g dry defatted tissue N number of animals The numbers in brackets are s e m

TABLE 2

The Influence of Thyroxine on Granulation Tissue of Normal Rats

	n	Weight of wet tissue	Weight of dry, defatted tissue	Relative water content	Hydroxyproline	Collagen
Control	12	396 (20)	54 (2)	618 (13)	2360	17 61 (0 38)
Thyroxine treated	12	455 (26)	63 (3)	622 (12)	2607	19 45 (0 55)*

* P = 0 02

For explanation see Table 1

different from the controls The author has not dared consider a value of $P = 0.02$ as a significant sign of increase of the collagen concentration It is clearly seen from the tables that treatment with thyroxine in rats does not have effects comparable to those seen in the guinea pig

Histological Descriptions

Below the crusta the tissues investigated are composed of an upper layer infiltrated with inflammatory cells and a deeper layer exclusively composed of fibroblasts and collagenous fibrils The present investigation is mainly concerned with this deeper layer

Control wounds The fibroblasts of this group have a great tendency towards a linear arrangement They assume many different shapes, some being stellate, others spindle shaped or round Most of the cells contain a nucleus with a moderate amount of chromatin, some, however, contain more darkly or more lightly staining nuclei The fibroblasts are placed in a rather sparse ground substance containing numerous collagenous fibrils and capillaries The ground substance gives a weak, positive reaction both with alcian blue and the Hale technique as well as staining metachromatically with azur A after treatment with pepsin Without this pre-treatment no metachromasia is found The ground substance also reacts weakly positively with PAS Mast cells are exceedingly rare (Fig 1)

Thyroxine treated The amount of intercellular substance is not changed and no reduction of the intensities of the Hale or alcian blue reactions is seen Nor is the amount or appearance of the collagenous fibrils changed Many of the fibroblasts contain a nucleus with a decreased amount of chromatin These cells do not show the orderly arrangement seen in the control group Areas of normal appearing fibroblasts, however, are also seen (Fig 2)

Ascorbic acid deficient group In this tissue very few capillaries are seen and extravasations of erythrocytes are found everywhere The

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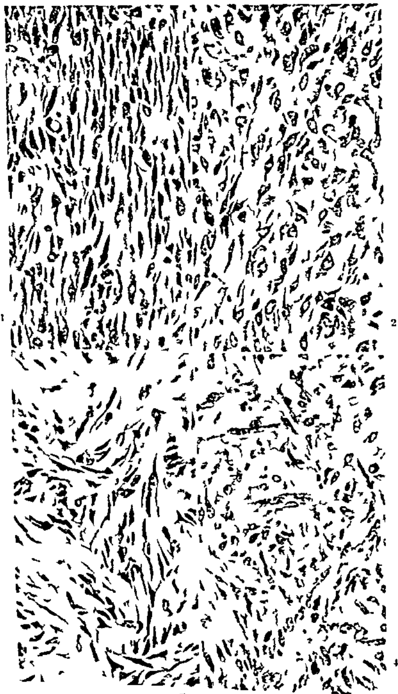
Figs 1 4

Fig 1 Normal granulation tissue Haematoxylin eosin staining Magnification 350 Note orderly arrangement of fibroblasts and relatively sparse intercellular substance

Fig 2 Granulation tissue treated with thyroxine Haematoxylin eosin staining Magnification 350 Note loss of orientation of the fibroblasts The nuclei are stained more lightly than in the control group

Fig 3 Granulation tissue from ascorbic deficient animal Haematoxylin eosin staining Magnification 350 The fibroblasts appear to lie quite chaotically in the tissue Extravasations of erythrocytes are seen in the abundant intercellular substance

Fig 4 Granulation tissue from ascorbic acid deficient animal treated with ascorbic acid for 2 days Haematoxylin eosin staining Magnification 350 The amount of intercellular substance has decreased the nuclei of the fibroblasts are more lightly stained and the breeding has disappeared



Figs 1 3

intercellular substance is considerably increased in amount and gives a very strongly positive reaction with the alcian blue and Hale methods The metachromasia after treatment with *pepsin is increased* The PAS-reaction, too, is more intense than in the control wounds The fibroblasts are present in normal numbers They are placed quite chaotically in the tissue *Two forms of fibroblasts seem to exist One of these* contains an ample amount of cytoplasm and a very lightly staining nucleus whereas the other one only contains little cytoplasm and a pycnotic nucleus Practically no collagenous fibrils are seen (Fig 3)

Short term ascorbic acid treatment· Treatment with ascorbic acid for two consecutive days before death results in great regressions of the scorbutic changes in the tissues Among the most conspicuous alterations are the disappearance of the bleeding, a considerable formation of collagenous fibrils, and a great reduction of the amount of intercellular substance The mucopolysaccharide staining reactions-including the PAS-reaction – are reduced in intensity The fibroblasts retain most of their disorganized arrangement, but a striking change of their morphology is seen The cells containing the pycnotic nuclei have disappeared and the fibroblasts as a whole are of a rather uniform appearance Most of the nuclei contain only small amounts of chromatin (Fig 4)

Thyroxine treated ascorbic acid deficient group The amount of intercellular substance is reduced very much as are the intensities of the Hale and alcian blue reactions The changes of these reactions are comparable to those found after the short term treatment with ascorbic acid apart *from* the *fact that no reduction* is seen *of* the intensity of the PAS-reaction In spite of the changes cited, the general impression is clearly scorbutic with extravasations of erythrocytes and no formation *of capillaries or collagenous fibrils The fibroblasts lie quite* disorganized, most of them containing an ample amount of cytoplasm and very pale nuclei, a considerable fall in the number of fibroblasts with *pycnotic nuclei having taken place* (Fig 5)

In the granulation tissue of all groups—especially in the ascorbic acid deficient animals—cells containing Hale-positive granules are seen *These granules do not react metachromatically with azur A, nor* do they stain with alcian blue When the Hale procedure is performed with omission of the colloidal iron reagent, a positive reaction is still seen This seems to indicate that *these cells are siderophages engaged* in the removal of extravasated erythrocytes Most of these cells contain a round, excentrically placed nucleus and an ample amount of cytoplasm In many instances, however, *transitional forms resembling* fibroblasts are seen

Enzyme incubations In all groups af granulation tissues the intensities of the Hale and alcian *blue* reactions *are reduced when* the staining reactions are made after incubation with streptococcal hyaluronidase, incubation with testicular hyaluronidase, however, abolishes the

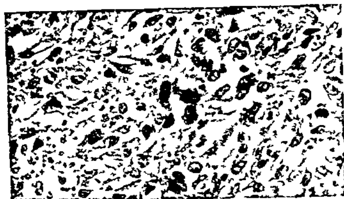


Fig 5

ranulation tissue from ascorbic acid deficient animal treated with thyroxine haematoxylin eosin staining Magnification 350 The pycnotic nuclei of the granulation tissue of the untreated ascorbic acid deficient animal have been replaced by more lightly stained nuclei The amount of intercellular substance is reduced in amount but no reduction of the extravasations of erythrocytes is seen

reactions completely The PAS reaction is not influenced by incubation with hyaluronidase

DISCUSSION

As shown by *Moltke* (19) administration of racemic thyroxine results in a decrease of the tensile strength of linear wounds in the guinea pig This result is in keeping with the decreased production of granulation tissue during treatment with thyroxine (Table 1)

Some previous investigations into the effect of thyroid hormones on wound healing have been made on rats and have given somewhat divergent results *Barclay et al* (2) found the healing to be promoted in open wounds by treating the animals with an extract of the thyroid gland in spite of the fact that this treatment led to a fall of the weight of the animals In agreement with this result *Desaulles et al* (80) found an increased weight of foreign body granulomas during treatment with thyroxine *Chassin et al* (5) however measuring tensile strength of laparotomy wounds found this to be reduced by thyroxine As at the same time the weights of the adrenal glands were increased, they advanced the hypothesis that the subdued healing was caused by a stress reaction As is seen from table 2 a considerable difference is found of the susceptibility of the rat and guinea pig to thyroxine The results seem to indicate that the rat is not influenced by this compound by the doses used here

The histological picture of regenerating tissue during treatment with thyroid hormones has not been investigated previously in the guinea pig In turpentine abscesses in the rat treatment with thyroxine did not result in histological changes (*Taubenhaus et al* (28)), whereas a reduction of the amount of collagenous fibrils is seen in intraperitoneal

intercellular substance is considerably increased in amount and gives a very strongly positive reaction with the alcian blue and Hale methods. The metachromasia after treatment with pepsin is increased. The PAS reaction, too, is more intense than in the control wounds. The fibroblasts are present in normal numbers. They are placed quite chaotically in the tissue. Two forms of fibroblasts seem to exist. One of these contains an ample amount of cytoplasm and a very lightly staining nucleus whereas the other one only contains little cytoplasm and a pycnotic nucleus. Practically no collagenous fibrils are seen (Fig. 3).

Short term ascorbic acid treatment. Treatment with ascorbic acid for two consecutive days before death results in great regressions of the scorbutic changes in the tissues. Among the most conspicuous alterations are the disappearance of the bleeding, a considerable formation of collagenous fibrils, and a great reduction of the amount of intercellular substance. The mucopolysaccharide staining reactions-including the PAS-reaction – are reduced in intensity. The fibroblasts retain most of their disorganized arrangement, but a striking change of their morphology is seen. The cells containing the pycnotic nuclei have disappeared and the fibroblasts as a whole are of a rather uniform appearance. Most of the nuclei contain only small amounts of chromatin (Fig. 4).

Thyroxine treated ascorbic acid deficient group. The amount of intercellular substance is reduced very much as are the intensities of the Hale and alcian blue reactions. The changes of these reactions are comparable to those found after the short term treatment with ascorbic acid apart from the fact that no reduction is seen of the intensity of the PAS-reaction. In spite of the changes cited, the general impression is clearly scorbutic with extravasations of erythrocytes and no formation of capillaries or collagenous fibrils. The fibroblasts lie quite disorganized, most of them containing an ample amount of cytoplasm and very pale nuclei, a considerable fall in the number of fibroblasts with pycnotic nuclei having taken place (Fig. 5).

In the granulation tissue of all groups—especially in the ascorbic acid deficient animals—cells containing Hale-positive granules are seen. These granules do not react metachromatically with azur A, nor do they stain with alcian blue. When the Hale procedure is performed with omission of the colloidal iron reagent, a positive reaction is still seen. This seems to indicate that these cells are siderophages engaged in the removal of extravasated erythrocytes. Most of these cells contain a round, excentrically placed nucleus and an ample amount of cytoplasm. In many instances, however, transitional forms resembling fibroblasts are seen.

Enzyme incubations. In all groups of granulation tissues the intensities of the Hale and alcian blue reactions are reduced when the staining reactions are made after incubation with streptococcal hyaluronidase, incubation with testicular hyaluronidase however abolishes the

reduction of the amount of mucopolysaccharides As at the same time the relative water content is diminished it seems likely that treatment of scorbutic granulation tissue with thyroxine results in a normalizing effect of the concentration of hyaluronic acid hyaluronic acid being the mucopolysaccharide thought to be responsible for the water binding of connective tissue (conf e g *Hvidberg & Jensen* (13))

The author thus feels justified to advance the hypothesis that treatment with thyroxine is able to correct some of the pathological changes of the ground substance of healing wounds in animals deficient of ascorbic acid Other aspects of wound healing are not corrected however Thus the collagen formation is not influenced the defects of the capillaries are not repaired and the fibroblasts—although altered—are not normalized

SUMMARY

The influence of thyroxine on granulation tissue formation of normal and ascorbic acid deficient guinea pigs has been investigated In normal animals this treatment results in a decrease of the amount of tissue formed without concomitant changes of the collagen content In animals deficient in ascorbic acid the increased amount of tissue formed and its water content is reduced by treatment with thyroxine In these animals the increased amount of intercellular substance is reduced as are the mucopolysaccharide staining reactions These changes resemble those found after a short term treatment with ascorbic acid The morphology of both normal and ascorbic acid deficient fibroblasts is changed by treatment with thyroxine

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quartz granulomas in mice (*Schiller* (26)) In the present investigation no changes are seen of the collagenous fibrils in spite of the fact that alterations are found both of the arrangement of the fibroblasts and of their morphology during treatment with thyroxine This finding is in agreement with the fact that the concentration of hydroxyproline is not influenced by treatment with thyroxine (Table 1)

Although the healing processes are seriously impaired in ascorbic acid deficiency, it is generally recognized that the fibroblasts exist in great numbers in scorbutic granulation tissue All authors, however, have reported great changes of their morphology Thus, according to *Hunt* (12) the fibroblasts of scorbutic granulation tissue contain a vesicular, lightly staining nucleus This was also reported by *Bunting & White* (4) whereas the cells described by *Penney & Balfour* (23) contained rather pycnotic nuclei All authors agree that practically no collagenous fibrils are seen in scorbutic granulation tissue

Because of the increased fragility of the capillaries, considerable bleeding is a common finding in scorbutic granulation tissue—as in scurvy in general Apart from the bleeding, changes of the mucopolysaccharides of the ground substance seem to be very characteristic of the healing processes during ascorbic acid deficiency As already mentioned, biochemical investigations indicate an accumulation of hyaluronic acid in granulation tissue of scorbutic animals (*Robertson & Hinds* (24), *Slack* (27), *Jorgensen* (16)) The amount of sulphated mucopolysaccharides, however, seems to be reduced (*Kodicek & Loewi* (17), *Moltke* (20), *Hughes & Kodicek* (11)) All workers using histochemical methods also have found great changes of the mucopolysaccharide content of scorbutic granulation tissue There is not, however, complete agreement as to the exact nature of the alterations found Thus metachromasia is both described as being decreased (*Penney & Balfour* (23), *Dunphy et al* (9)) and increased (*Bunting & White* (4), *Levenson et al* (18) A possible explanation of the divergent results has been given by *Jorgensen* (15) In the present investigation it has been possible to remove part of the positive staining reaction with alcian blue and the Hale procedure by means of streptococcal hyaluronidase whereas treatment with testicular hyaluronidase is able to abolish the reactions completely This finding signifies the presence of both hyaluronic acid and chondroitin sulphate

Only little is known about the possible changes of the ground substance and its content of mucopolysaccharides when animals bearing wounds are treated with thyroid hormones In linear, incised wounds a reduced incorporation of radioactive sulphate has been found (*Moltke* (20)) In the present investigation treatment with thyroxine does not seem to influence neither the relative water content nor the histochemical reactions of normal granulation tissue When, however, scorbutic granulation tissue is treated with thyroxine, a significant reduction of the intensity of the staining reactions is seen This indicates a

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METASTASIZING BASAL-CELL CARCINOMA

By

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Received 14. v. 63

The term basal-cell carcinoma or basal-cell epithelioma designates a skin tumour which is manifested in several different forms. In its pure form without differentiation into squamous epithelium it is considered by many to be unable to set metastases. In the course of the years, however, several cases of basal cell carcinoma which have metastasized to lymph glands and even to parenchymatous organs have been described. In 1951 Lattes and Kessler listed the cases that had been reported up to that time. Together with two cases described by themselves, twenty acceptable cases had then been published. In recent years, however, further cases have occurred. If the criteria given below are applied, a scrutiny of the relevant literature shows that 36 well-documented cases have been described. These are tabulated in Table 1.

Lattes & Kessler (1951) and *Cotran* (1961) have set up definite criteria for metastasizing basal-cell carcinoma.

(1) The primary tumour must be localized to the skin.
(2) It must be possible to show metastases in lymph glands or viscera.
(3) The histopathological picture in both the primary tumour and the metastasis must show the typical form of basal-cell carcinoma. There must be no signs of epidermoid differentiation.

The majority of the cases listed in Table I show a very long period of time from the appearance of the primary tumour until the date when it has been possible to show metastases. In several cases no definite indication of the period has been given, but as an approximate mean for the whole material ten years seems to apply. There is no definite difference with reference to the sexes.

At the Oto-laryngologic Clinic and the Pathological Institution of Karolinska sjukhuset we have had an opportunity of examining more closely a case of metastasizing basal-cell cancer.

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Fig. 1
Postmortem picture of the face

Autopsy Finds

The external inspection showed an old woman with very strongly reduced flesh. Almost the entire right side of the face was destroyed by an in part necrotizing tumour eroding over on the underlying bone tissue. The right orbit was infiltrated with tumourous tissue but no definite growing over onto the atrophied bulb was observed. The skin was otherwise pale and no further tumours could be observed on the same.

Thoracic organs.—The lungs were rather heavy and oedematous. In the right pleural cavity was about 200 cc exudate which had caused marked compression atelectases in the lung. There were slight tracheo-bronchitis and minor bronchopneumonias. No tumour suspect foci were observed. There were no pathological changes in heart and vessels.

Abdominal organs. The peritoneal surfaces were normal. The liver was of normal size but several small abscess foci and a couple of super

TABLE 1

Author		Site of primary tumour	Localization of metastasis
Beadles	1894	face	lymph node
Fordyce	1902	nose	lymph node
Körbl (Case 61)	1912	temple	lymph node
Hazen	1917	hand	lymph node
Hazen	1917	nose	lymph node
Hazen	1917	right axilla	lymph node
Hazen	1917	forehead	lymph node
Finnerud	1924	face	lymph node
Finnerud	1924	face	lymph node
Mulzer	1930	temple	lymph node
Mulzer	1930	temple	lymph node
Spies	1930	cheek	bones, lungs, spleen, liver
De Navasquez	1941	forehead	lungs, bones
Singer	1945	face	bones
Streitmann	1946	face	lymph nodes
Amersbach	1947	scalp	lymph node
Foot	1947	face	lymph nodes
Small	1949	ear	lung, liver, spleen, kidney, pleura, peritoneum, lymph node
Small	1949	ear	lung, liver, spleen, kidney
Fekhoff	1951	scalp	lymph node
Lattes & Kessler	1951	wrist	lymph nodes
Lattes & Kessler	1951	scalp	lymph nodes
Huntington & Levan	1957	face	lung
Richter	1957	scrotum	lung
Pickren & Katz	1958	face	lung (aspiration)
Pickren & Katz	1958	face	lung (aspiration)
Cotran	1961	scalp	lymph node
Cotran	1961	face	lymph node, lung
Cotran	1961	scalp	lymph node
Cotran	1961	eyelid	left parotic region
Cotran	1961	face	lymph nodes
Cotran	1961	nose	lymph nodes
Cotran	1961	scapular region	lymph node, lung
Cotran	1961	eyelid	lymph node
Cotran	1961	scalp	lymph node
Raitschev et al	1961	scalp	ear

DESCRIPTION OF CASE

The patient was a 61-year old woman who for at least three years had had a growing tumour on the right side of her face The tumour had probably started as a small ulceration under the right eye She had never been troubled by any severe pain, but for at least a year she had been unable to see with her right eye In the course of the years the tumour had destroyed and ulcerated practically the whole of the right side of the face An increasing trismus had for a long time rendered nutrition difficult and in the last few months she had only been able to consume liquid food On account of "fear of the doctor she had not wanted to seek help earlier

On admittance to the Oto-laryngologic Clinic the patient was extremely pale and thin The whole of the right side of her face was destroyed by a large ulcerating tumour extending from the forehead right down over the mandible In the ravaged area projected an atrophied remainder of the bulb The orbit was in part destroyed and the maxillary sinus was perforated (Fig 1) The patient had a slight subnormal temperature There were no clinical signs of sepsis There was no time to make a closer examination, as the day after admittance the patient went quietly ad mortem



Fig 4
Hæm eos × 130

Fig 5
Hæm eos × 274

Figs 4-5
Metastases in the liver from the basal cell carcinoma

ficially located tumourous formations were found in it These latter were grey white with homogeneous section surfaces The foci attained a diameter of 1 cm The spleen was to a large extent destroyed by abscesses The pancreas was ordinary It was not possible to show any pathological changes in the gastrointestinal canal

The urogenital organs were normal apart from signs of senile atrophy of the genitals

No pathologically changed lymph glands were shown in the course of the autopsy

Microscopic examination —In sections from the tumour in the right side of the face was seen a typical basal cell carcinoma without signs of epidermoid differentiation (Figs 2 and 3) The tumour foci in the liver showed in principle the same picture as the primary tumour Figs 4 and 5) The tumour grew in fine strands built up of basal cells In several places moreover was seen a layer of palisade set cells

In sections from other organs were extensive inflammatory changes of the type found in connection with sepsis It was not possible to show histopathologically any further tumourous changes or metastatic foci



Fig 2
Htx eos × 130



Fig 3
Htx eos × 224

Figs 2 3
Skin Typical basal cell carcinoma

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SEROLOGICAL STUDIES ON HAEMOPHILUS INFLUENZAE AND RELATED SPECIES

3. *Adaptation of the Gel Precipitation Method to the Study of Type Specific Antigens*

By

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In a previous article results were reported from the examination of a material of H. influenzae strains by means of the capsular swelling reaction (*Omland* 1963b). These results were intended as a basis of further serological studies of superficial antigens, especially antigens of type specific nature. For this task a suitable serological technique had to be selected and adapted.

As documented by an extensive literature on the serology of these organisms, agglutination procedures have proved difficult to interpret. Representative works on the subject have been reviewed in an earlier study (*Omland* 1963a).

Other methods, such as complement fixation and indirect haemagglutination, are considered unsuitable for the examination of type specific antigens (*Tunevall* 1953b, *Warburton, Keogh & Williams* 1949, *Glynn* 1959).

However, precipitation technique has been employed successfully in a number of studies on such antigens (*Pittman* 1931, *Platt* 1937, *MacPherson* 1948).

The method of precipitation in gel in particular has been used in a series of works on the antigenic structure of Haemophilus and related genera. Thus various species of Pasteurella have been studied by means of gel precipitation (*Maclennan & Rondle* 1957, *Daod Nathoo, Dodin & Brygoo* 1959, *Lawton, Fukui & Surgalla* 1960). Brucella and other Parvobacteriaceae have been examined by this technique (*Olitzki* 1959, 1960, *Olitzki, Godinger & Sharon* 1959). The method has also been used in investigations of non-type-specific H. influenzae antigens by *Tunevall* (1953a), and in comparative serological studies on H. influenzae and H. aegyptius by *Olitzki & Sulitzeanu* (1959).

The gel precipitation technique has proved a great methodological advance in microbiology and immunology in general, thanks to a number of fundamental studies (*Oudin* 1948a, b, *Ouchterlony* 1948, 1949a, b,

SUMMARY

In connection with an account of a case of metastasizing basal cell carcinoma a scrutiny of the relevant literature has been made From this it has emerged that 36 well documented cases have been published These were chiefly very advanced primary tumours and a long period had elapsed from the appearance of the primary tumour until it was possible to show metastases, on an average ten years

The case described here was that of a 61-year old woman who for at least three years had had an ulcerating basal-cell cancer on her right cheek The patient died on account of sepsis The autopsy revealed metastases of basal-cell carcinoma in the liver

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b) *Establishing Reference Systems of Known Serological Types (Large-Scale Method)*

Antigens The antigens were prepared according to the principles described by *MacPherson* (1948) Because certain modifications have been made, the procedure followed shall be presented in detail· A chocolate agar plate (heated horse blood agar with added ascitic fluid) was inoculated heavily with a pure culture of the strain under test After 18 hours' incubation in a moist chamber at 37° C the culture was harvested in a minimum (about 1 5 ml) of 1 M sodium acetate and precipitated by 5 volumes of ethanol (95 per cent) The mixture was left in the refrigerator (+4° C) for 4 5 hours or until the following day, and was then centrifuged at the same temperature and moderate speed (1000–1500 g) for 30 minutes The supernatant was discarded and the precipitate dissolved in 1 ml of 90 per cent phenol and left at room temperature for 15 minutes One tenth millilitre of 20 per cent sodium acetate was added, and thereafter 5 volumes (5 5 ml) 95 per cent ethanol for reprecipitation The mixture was placed in ice water for a minium of 1 hour After centrifugation at the same speed and temperature as described above, the supernatant was discarded and the precipitate washed 2–3 times in 95 per cent ethanol Finally the precipitate was extracted over-night in 1 ml of distilled water or 0 85 per cent saline at room temperature The antigen solution thus obtained was used for the precipitation experiments

Further modifications of the procedure have been employed elsewhere

TABLE 1

Gel Precipitation Experiments (Large-Scale Method) with Type Antigens and Antisera in Various Combinations

Serum	Immunization period	Antigen	Property of	
			type specific line(s)	non-type-specific line(s)
A	short	a	weak	moderate
A (—3)	long		weak	strong
B	short	b	moderate	moderate
B (—5)	long		moderate	strong
C	short	c	moderate	moderate
C (—7)	long		moderate	strong
D	short	d	absent	trace
D (—8)	long		trace	trace
F	short	e	strong	trace
F (—9)	long		strong	weak
F (—10x)	long	f	trace	weak

The serum symbols A B C, D and F represent serum portions drawn before the conclusion of the immunization periods from the same animals as the corresponding A B C D, and E

and *Elek* 1949) Since the time of these classical works numerous studies have been dedicated to the development of this technique, both as concerns theoretical and practical aspects It is, above all, in qualitative studies of complex serological systems that the method has confirmed its value, and here notably in the form of the so-called double diffusion arrangement The extensive work aimed at developing the gel precipitation into a quantitative technique has, up to the present time, led to few results Some of the more recent studies on the method must be mentioned: *Ouchterlony* (1958), *Wilson* (1958), *Aladjem, Jaross, Paldino & Lackner* (1959), *Becker & Hayden* (1960), *Buchanan-Davidson & Oudin* (1960), *Darcy* (1960).

EXPERIMENTAL

The purpose of this part of the present study was to develop a modification of the gel precipitation method, suitable for use in the study of type specific and other H influenzae antigens

a) *Gel Precipitation Systems in Petri Dishes* (*Large-Scale Method*)

A modification of the Petri dish method of agar gel double diffusion was adapted during the initial phase of this study, and is hereby described in detail The gel was prepared from 1 5 per cent Difco agar in unbuffered saline (0 85 per cent), and poured into Petri dishes with a diameter of 85–90 mm A 1–2 mm layer was poured and allowed to harden A slip of paper with the design of the hole pattern was then placed under the dish, and penicillin assay cylinders (8 mm external diameter) were placed accordingly upon the agar Another layer of agar (about 5 mm) was then poured and left to harden The cylinders were removed by means of pincers, making a slight rotating movement to avoid damaging the edges of the holes thus prepared A levelled table was used for the work The most common pattern consisted of one central and six peripheral holes with a distance between neighbouring holes of 6 mm (edge to edge)

Based on preliminary experiments the following conditions were chosen for the precipitation experiments After filling antigen and serum into the holes, the systems were incubated in a moist chamber for 5 days at room temperature Longer incubation (up to 10 days) yielded no additional information No exact quantitation of antigen and serum was attempted, as it is generally accepted that concentration gradients rather than absolute volumes are basic factors in gel precipitation systems (*Becker, Munoz, Lapresle & LeBeau* 1951, *Neff & Becker* 1957, *Aladjem, Klostergaard & Paldino* 1960) The reactants were simply filled up to the rim of the holes as uniformly as possible

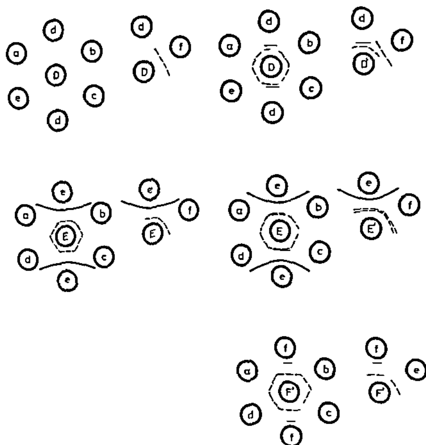


Fig 1

is apt to increase the content and variety of non type specific antibodies in the serum

c) *Adjustment of the Concentrations of Reactants (Large Scale Method)*

If within the range of concentrations giving visible precipitation—a series of experiments is set up keeping one reactant at constant concentration while lowering the concentration of the other, the precipitate line (or lines) will form successively nearer to the hole containing the latter reactant (*Ouchterlony* 1958) The curvature of the precipitate line will also be influenced by concentration changes but is more specifically determined by the diffusibility ratio between the two reactants, tending towards concavity in relation to the hole containing the least diffusible reactant (*Korngold & van Leeuwen* 1957, *Aladjem Jaross, Paldino & Lackner* 1959)

The concentrations of reactants are thus important basic conditions

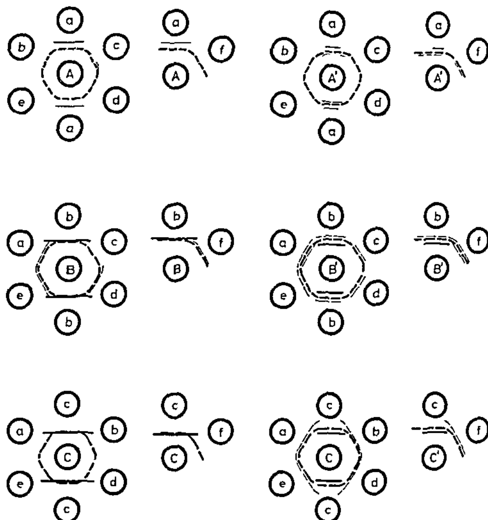


Fig 1

Gel precipitation experiments (large scale method) with type antigens and antisera in various homologous and heterologous combinations showing type specific (continuous lines) and non-type specific precipitates (dotted lines) For explanation of symbols see Table 1

in this series of Haemophilus studies, and will eventually be described (*Omland* 1963c)

Antisera The antisera were prepared according to descriptions presented earlier (*Omland* 1963b)

Some of the experiments carried out in the large-scale gel precipitation method for establishing type reference systems are summarized in Table 1

Fig 1 shows examples of the type reference systems listed in Table 1

The experiments showed that reproducible type reference systems could be established The quality varied, however, from one system to another Thus the systems of the types a, b, c, and e yielded distinct results, while types d and f yielded very weak type specific precipitations Fig 1 also shows the effect of prolonged immunization, which

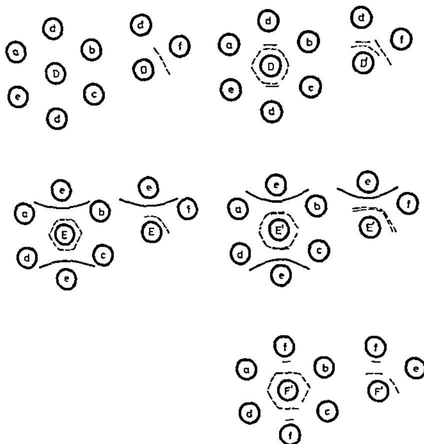


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The concentrations of reactants are thus important basic conditions

which have to be adjusted in each particular system. Because the antibody contents of the sera are in general limiting factors in precipitation experiments, it is advisable to use the sera undiluted, and consequently adjustments of concentrations have to be made in the antigen solutions.

Based on these considerations experiments were designed varying the antigen concentrations in the respective type reference systems.

The antigen solutions were prepared from dried, type specific polysaccharide standards. These substances were prepared by fractionated ethanol precipitation. The procedure will be described in a subsequent article (*Omland* 1963c).

A stock solution was prepared in 0.85 per cent saline from each standard antigen. The concentrations (1 mg per ml for all antigens) were chosen arbitrarily, and from the stock solutions twofold dilution series were prepared and filled into the peripheral holes in the agar in a clockwise sequence. The homologous antiserum was filled into the central hole. The antigen concentration giving barely visible precipitation, and the concentration providing optimal conditions for observation and recording, were registered in each system. The results are presented in Table 2. Fig. 2 serves as an illustration to the titration procedure.

TABLE 2

Concentrations of Type Specific Antigens Yielding barely Visible respectively Maximally Distinct Precipitations with Homologous Antisera (Large Scale Method)

Type	Serum	Concentrations of type specific antigens for: barely visible precipitation	Concentrations of type specific antigens for: maximal distinction for recording
a	3	0.25 mg/ml	0.5 1.0 mg/ml
b	5	0.004 mg/ml	0.03 – 0.25 mg/ml
c	15	0.016 mg/ml	0.06 0.25 mg/ml
d	16	0.125 mg/ml	0.25 – 1.0 mg/ml
e	9	0.062 mg/ml	0.25 1.0 mg/ml
f	10x	0.125 mg/ml	0.25 1.0 mg/ml

d) *Development of a Gel Precipitation Method in Small Scale on Slides (Miniature Method)*

At this stage of the work it was not known whether the difficulties encountered in certain type systems such as d and f (see Table 1), were mainly due to the antigen preparations or to the antisera. It was, however, obvious that extensive work had to be carried out to try to increase the distinctness of the type specific precipitations and to exclude non-type specific phenomena. As soon as this was realized, it became apparent that the problem of securing sufficient amounts of reactants would be one of the decisive factors of the whole work. In order to economize, it seemed thus most rational to try to develop a procedure where very small amounts would be sufficient.

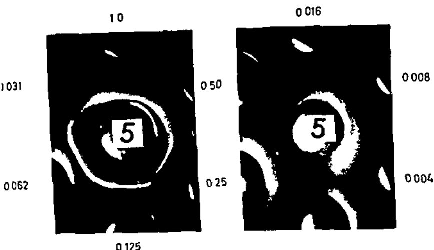


Fig 2

Titration experiment for adjustment of the concentration of type reference antigen type b (indicated values in mg/ml) against homologous undiluted serum (no 5)

Exploratory work was performed on techniques similar to those described by *Wadsworth* (1957) and *Crowle* (1958) using a perforated plexiglass matrix placed into exact contact with the agar surface The holes in the matrix represented the reagent reservoirs The experiments demonstrated the occurrence of undesirable precipitation phenomena at the contact surface and the technique was abandoned

The procedure finally adopted was based on the principles described by *Mansi* (1958) It consisted of preparing the small scale hole pattern in agar gel poured onto microscopical slides (76 × 26 mm) A device was constructed serving at the same time as a holder for the slide and a mould for two separate 7 hole patterns After the slide had been fixed and the bar (Fig 3) placed in the proper position the melted agar was poured directly onto the slide and allowed to harden The position of the bar had been chosen so as to obtain a continuous layer of agar also at the bottom of the holes and to prevent a direct communication down to the glass surface Thus only one operation was needed and the gel was ready for the precipitation experiment within 15 minutes from the pouring even though trimmings of the hole bottoms had often to be made using a looped fine-gauge metal wire mounted on a piece of cork An illustration of the moulding device is shown in Fig 3

The following dimensions were employed hole diameter 3 mm distance between edges of neighbouring holes 3 mm depth of holes 1 5 2 mm The reactants were filled into the holes by using drawn out finely tipped Pasteur pipettes which were filled by capillary suction holding the tip immersed in the reactant The amount desired could be controlled by holding the pipette more or less askew It could be emptied by pressing the thumb against the upper opening The holes were

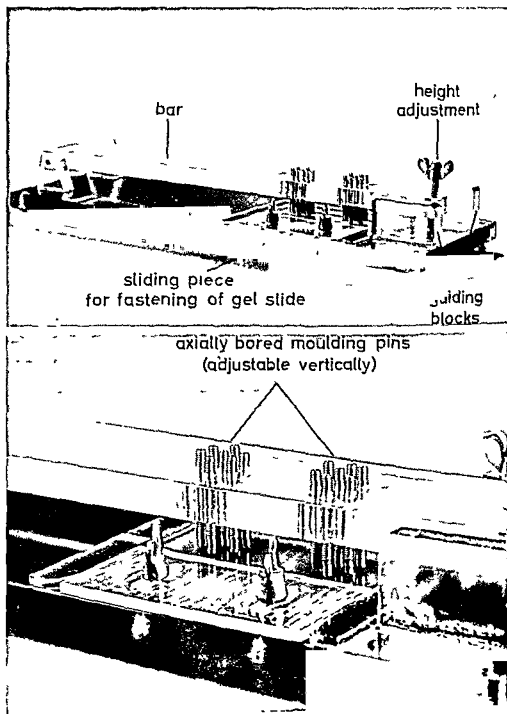


Fig 3
Device for the preparation of gel precipitation slides (miniature method)

filled up to the rim, leaving a moderately concave surface meniscus Further attempts at quantitating the reactants were not made for reasons already discussed under the large-scale method

It was important even in the large-scale method to secure enough

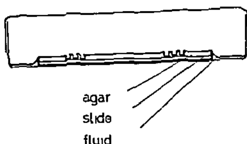


Fig 4
Moist chamber for gel precipitation (miniature method)

moisture during the incubation, and in the miniature method, with the greatly increased surface/volume ratio, drying phenomena tended to appear already after few hours if sufficient humidity was not present This could however be ensured by incubating the gel slides in a shallow layer of fluid of the same composition as the fluid phase of the gel In practice this was done by placing the slide in a Petri dish as shown in Fig 4

This arrangement allowed free communication between fluid and gel through the edge of the latter

Advantages of the miniature method The advantages of this method as compared to the large scale method are mainly of two kinds *viz* as concerns the economy of reactants, and the time of incubation

The economy of reactants may be illustrated by a simple calculation of the volumes of the holes used in the the respective methods In the miniature method it may be calculated to about *10 mm³* (r = 1 5 mm, depth = 1 5 mm), whereas in the large scale method the corresponding figure is about *250 mm³* (r = 4 mm, depth = 5 mm)

The time of incubation may be estimated as a function of the diffused distance, being proportional to the square of this distance (*Ouchterlony* 1958)

From the theoretical considerations the incubation time needed in the miniature method might be expected to be only about ¼ of the one required in the large scale method This is in good agreement with the results shown in Table 3

Possible disadvantages of the miniature method were seriously considered particularly the question of poorer resolution of the precipitate bands and problems connected with photographical recording Direct comparison with the large-scale method revealed no real decrease in resolution An *apparent* impairment of resolution is caused by the small dimensions which may occasionally lead to a situation where the precipitate bands are not oriented exactly perpendicularly to the slide surface To avoid misinterpretations caused by oblique projections of the bands it was thus necessary always before photographing to observe the gel slides meticulously with a lens against dark background

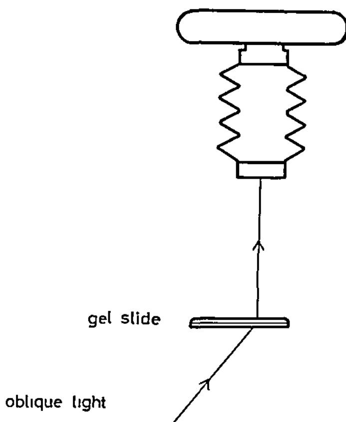


Fig 5
Photographical recording of gel precipitation

c) *Photographical Recording*

Technical problems concerning the photography of gel precipitations have been dealt with by several authors (*Hunter* 1959, *Reed* 1960)

The arrangement used in the present study for photographical recording is shown in Fig 5

Oblique light from one lamp was used Oblique light from several sources of light or in the shape of a light cone has been tried, however without leading to the same richness of detail as the former procedure The disadvantage of the used technique is the occurrence of some assymmetry, especially of refraction effects at the rim of each hole Misinterpretations, however, are easily avoided, as the effects occur regularly on one side of the hole

When large-scale negatives (90 × 120 mm) were needed, a special mirror reflex camera (Busch) was used However, for most purposes small negatives (24 × 36 mm) proved sufficient, and in these cases a miniature camera (Leica) was employed

High-contrast plates (Gevaert Replica 23) or films (Agfa Agepe) have been used, and a contrast developer has been found most suitable (*e g* Johnson Contr ›,t) Methylorange (3 100,000) was added to the

gel, as it improves slightly the contrast when orthochromatic negatives are used (*Björklund* 1952).

f) Variations in different Experimental Conditions (Miniature Method)

These examinations have been restricted to a limited number of factors considered relevant to the subsequent studies. Other basic conditions have been chosen arbitrarily. Thus room temperature was chosen for incubation, as it can easily be kept constant during the entire period of an experiment, and at the same time provides for a suitable rate of diffusion and precipitation.

The concentrations of reactants have been chosen according to considerations and findings presented above (cfr. c)). The sera have been used undiluted. The type specific polysaccharide standards have been used in the following concentrations: type a, 1 mg/ml, type b, 0.1 mg/ml, type c 0.1 mg/ml, type d, 1 mg/ml, type e, 1 mg/ml, type f, 1 mg/ml. The antigen solutions have been prepared in distilled water.

In these and later experiments a preservative (merthiolate "Lilly") has been added before pouring the gel, in a concentration of 1:10,000, together with a dye (cfr. e)).

1) *Time of incubation.* Reports on the comparatively short incubation periods needed in the miniature gel precipitation methods have been presented by *Wadsworth* (1957), and *Mansi* (1958).

Experiments on this point have been carried out with known homologous type systems, using 1.5 per cent agar and unbuffered 0.85 per cent saline in the gel. The systems were incubated for 5 days at room temperature and observed at short intervals during the first day, later daily. Precipitation could be observed already after 3–6 hours as barely visible opacities. After 24 hours the precipitation was distinct. After 48 hours the existing lines had become slightly more distinct, but no additional lines had appeared. After more than 2 days incubation the pattern of lines began to grow more diffuse. Even after 5 days, however, the main precipitation lines were discernible.

The results are shown in Table 3.

It was concluded from these experiments that the optimal incubation period would ordinarily be 2 days. Later experience confirmed these observations.

TABLE 3

Comparison of some Characteristics of the Large Scale and the Miniature Method of Gel Precipitation

Method	Hole distance (mm)	Incubation time until	
		barely visible precipitation	maximal distinction for recording
Large scale	6	ab. 1 day	5 days
Miniature	3	3–6 hours	1–2 days

2) *Agar concentration* During the preliminary work the same agar concentration was used as in the experiments on the large-scale method *i.e.* 1.5 per cent In these and all later experiments Difco Special Agar Noble has been used It might be expected that a reduction of the agar concentration would lead to a more transparent gel and thus make observations easier, and an experiment was set up to determine the lowest concentration at which the agar still possessed sufficient stability The following concentrations were tried 1.5, 1.2, 1.0, and 0.8 per cent As expected there was a considerable increase in transparency with reduced concentration The lowest agar concentration yielding necessary stability was 1.0 per cent, and this concentration has been adhered to in the subsequent studies

3) *Salt concentration and pH* Most workers have used physiological salt solutions, unbuffered or buffered at neutral pH However, *Olitzki* (1959) reported favourable results using a markedly hypotonic salt solution in gel precipitation studies on Brucellae

A series of experiments has been performed, varying the salt concentration and pH For this purpose mixtures of NaCl solutions and phosphate buffers have been employed The different mixtures are listed in Table 4

TABLE 4

Combinations of Salt Concentrations and pH Tried in Gel Precipitation Experiments on Homologous Type Systems (Miniature Method)

Salt concentration in mixture (mols)			pH measurements in mixture			
Total	NaCl	phosphates	buffer pH 6.0	buffer pH 6.5	buffer pH 7.0	buffer pH 7.5
0.01	0.005	0.005	6.05	6.50	7.00	7.55
0.02	0.01	0.01	5.95	6.50	7.00	7.50
0.05	0.04	0.01	5.90	6.50	7.00	7.50
0.10	0.09	0.01	5.85	6.35	6.80	7.30
0.15	0.14	0.01	5.80	6.25	6.80	7.30

TABLE 5

Influence of Variations in Salt Concentration and pH in Gel Precipitation Experiments on Homologous Type Specific Systems (Miniature Method)

Type	Serum	Antigen	Maximal distinction for recording at	
			salt concentration (mols)	pH
a	3	a (1 mg/ml)	0.01 0.05	6.5 7.0
b	5	b (0.1 mg/ml)	0.01 0.02	6.5
c	15	c (0.1 mg/ml)	all tested	6.0 7.0
d	16	d (1 mg/ml)	0.01 0.02	6.0 7.0
e	9	e (1 mg/ml)	0.01 0.05	6.5
f	10x	f (1 mg/ml)	?	?

In the lowest total concentration (0.01 M) a buffer concentration of 0.005 M has been used In all other instances the buffer concentration

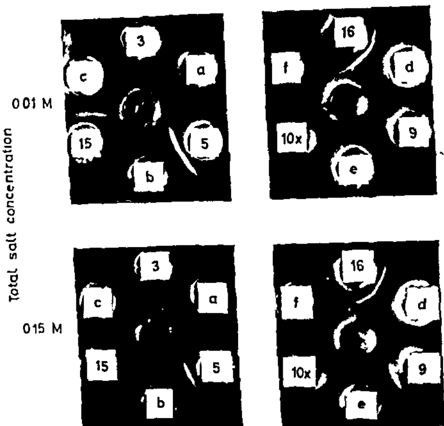


Fig 6

Gel precipitation experiments on homologous type systems showing the effect of variations in the total salt concentration The symbols used for antigens and antisera are the same as in Table 5

has been 0 01 M All pH values have been measured electrometrically The results are shown in Table 5

According to these results changes in salt concentration and pH tend to affect the various type systems in a somewhat different *manner* There is, however a clear trend to the effect that the *precipitations are* more distinct at low salt concentrations The effect of changes in pH is less obvious Though there is a tendency towards *more distinct* precipitations at pH 6 5 7 0 Fig 6 illustrates the difference between low (0 01 M) and high (0 15 M) salt concentration at an *intermediate* pH (6 2 6 5 see Table 4)

Based on these findings and considering the desirability of using only one salt buffer combination in the later work, it was decided that the best conditions for type specific standard systems would be a total salt concentration of 0 01 M and a pH of 6 8 (see Table 5)

DISCUSSION

In the search of a technique for the serological examination of a material of Haemophilus strains the gel precipitation method was chosen as the most suitable. The present study deals with the experimental adaptation of this method for the subsequent work.

The gel precipitation technique is essentially a comparative serological method, requiring a set of basic reference systems of antigens and antisera. A series of such systems had been established during the work on the capsular swelling reaction (*Omland* 1963b), and the first phase of the present study was aimed at confirming the reproducibility of the systems in gel, and at establishing a distinction between type specific and non-type-specific precipitation lines. During this work the best systems within each type could be selected.

Because the precipitation in the gel is governed by concentration gradients of antigens and antibodies, it was important to determine experimentally the optimal concentrations of reactants to be used. It was considered undesirable to dilute the antisera, as the antibody content is often a limiting factor in serological work of this kind, and the titrations of optimal concentrations were therefore performed in the antigens. This work was based on the use of dried standards of raw, type specific polysaccharides.

To prepare even small quantities of antigens from fastidious organisms like Haemophilus is costly in terms of media, time, and work. This applies also largely to the antisera when rabbits are used. It was obvious at an early stage that great difficulties would be met with in securing necessary amounts of reactants if conventional, large-scale gel precipitation was to be employed, and the only rational solution was apparently to develop a miniature modification. Not only would it be possible to economize with reactants, but even the incubation time could be greatly reduced.

A miniature gel precipitation method has then been established and described in detail. It fulfils the requirements of economy in the question of reactants and incubation time as mentioned above. Certain disadvantages, mainly caused by the small-size pattern, proved unimportant and could easily be compensated for by some experience. By photographical technique the results could be presented in any scale desired.

The last part of this study deals with the influence of variations in certain basic factors in the miniature method. The conditions judged optimal in work on type specific H influenzae systems are described: 2 days' incubation in 1 per cent agar at pH 6.5–7.0 using a salt concentration of 0.01 M.

SUMMARY

The experimental adaptation of the gel precipitation method for the study of type specific and other H. influenzae antigens has been studied.

The first part of the study has been performed in conventional large scale experiments aimed at confirming the reproducibility of each type system and at distinguishing type specific from non type-specific precipitation lines Distinct type specific systems were established in the types a b c and e Optimal antigen concentrations to be used in each type system were determined employing dried type specific polysaccharide standards

The subsequent part of the present study deals with the development of a miniature gel precipitation technique directly adapted to the study of a large number of strains Investigations have been carried out into certain basic conditions such as the concentrations of agar and salts and pH Based on these examinations the following conditions have been selected as optimal in the study of type specific systems 1 per cent agar a total salt concentration of 0 01 M and pH 6 5–7 0 The miniature gel precipitation method has great advantages in terms of economy of reactants and time of incubation

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FROM THE TUBERCULOSIS AND STATISTICAL DEPARTMENTS, STATENS SERUMINSTITUT, COPENHAGEN, AND THE NATIONAL INSTITUTE OF HEALTH, SEOUL, KOREA

TUBERCULIN PRODUCTION

3. *Tuberculin Yield with Different Strains of Mycobacterium tuberculosis*

By

Mogens Magnusson, Hyun Kyu Kim and M. Weis Bentzon

Received 7.iii.63

Several strains of *Mycobacterium tuberculosis* are used for preparation of tuberculin for practical purposes (*Seibert & Glenn* 1941, *Green* 1946, *Svenkerud* 1955, *Desbordes* 1958, *Magnusson & Bentzon* 1958). In Japan only one single strain is employed (*Ministry Hlth. Welf., Jap.* 1961) and in other places three to eight different strains. Among other properties the yield and specificity of the tuberculin obtained may be decisive factors in selection of strains for tuberculin production.

The aim of the present work is to compare the yield of tuberculin obtained with different strains of *M. tuberculosis*. The specificity of tuberculin derived from four of the strains will be considered in a subsequent work.

EXPERIMENTAL

The total crop of bacteria, yield of tuberculoprotein and tuberculin yield were compared for seven different strains of *M. tuberculosis* and the BCG strain[1], cultured on Sauton and Lind bII medium (*Sauton* 1912, *Lind* 1948). The strains were inoculated on to small flasks containing 180 ml medium. After incubation from one to nine weeks cultures were sterilized, after which the dry weight of organisms, pH and protein content of culture filtrate *in each flask* were measured. The tuberculin activity of some of the culture filtrates was assessed by intradermal tests on BCG vaccinated guinea pigs.

MATERIALS AND METHODS

Media. Sauton and Lind bII medium were prepared as prev[illegible] nusson, Kim & [illegible] sterilized by au[illegible]

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[1] BCG is an attenuated strain of *M. bovis*.

Strains *Aoyama B* is the strain of *M tuberculosis* used in Japan for tuberculin production The strain was received from Dr K Yanagisawa Tokyo in 1955

PN and C are strains of M tuberculosis used extensively for tuberculin production (*Green* 1946) These were received from Dr A B Paterson Weybridge, England in 1955

E5 F9656 and U1921 are virulent strains of *M tuberculosis* isolated from Danish tuberculosis patients more than 10 years prior to the present study see *Lind* (1948)[1] The strains were among those used for preparation of purified tuberculin RT23 (*Magnusson & Bentzon* 1958)

T3505 is a virulent strain of *M tuberculosis* freshly isolated from a Danish patient at the time of the experiment

BCG The culture of BCG is the strain used in the BCG Department Statens Serum institut for preparation of BCG vaccine

Two 17 day old cultures of each strain grown on 180 ml Sauton medium were used for inoculation of the test cultures on to Sauton medium Similar 18 day old cultures were used for inoculation on to Lind bII medium A pellicle with a diameter of about 10 mm was used as inoculum for each flask and the cultures were incubated at 38° C One or more flasks of each strain were sterilized by heating in streaming steam for one hour at weekly intervals up to 9 weeks after inoculation

Measurement of the dry weight of organism pH and protein content of culture filtrate in each flask was made as described previously (*Magnusson Kim & Bentzon* 1963)

Tuberculin activity of culture filtrates The tuberculin activity of the culture filtrates was measured for each strain using the flask which showed the largest protein content In a few cases however sterile samples of these cultures were not available and flasks with slightly lower tuberculoprotein content were used The tuberculin activity of the filtrates was compared with reference dilutions containing *100 TU and 10 TU per 0 1 ml by intradermal testing in BCG vaccinated guinea pigs* Dilutions of purified tuberculin (PPD) RT22 Statens Seruminstitut containing 0 0017 mg (100 TU) and 0 00017 mg (10 TU) per 0 1 ml were used as reference dilutions

Tuberculin dilutions On the basis of preliminary assays it would be expected that reactions of a size between those given by the two standard doses might be obtained with the culture filtrates after dilution 1 200 Fresh dilutions 1 200 were therefore prepared from the culture filtrates on each testing day using phosphate buffered saline pH 7 38 as diluent

Experimental animals Forty eight albino guinea pigs bred at the farm attached to Statens Seruminstitut were used Each animal was vaccinated intracutaneously in four places on the abdomen with 0 1 ml of BCG vaccine[2] containing 7 5 mg BCG bacteria (semi dried weight) per ml On the vaccination day the animals weighed 450 570 grammes The vaccines (batches Nos 1151 and 1152) were used when freshly prepared The animals were used two to three weeks after vaccination

Experimental design The eight culture filtrates from Sauton medium were n lected together with the standard dilutions into groups of eight guinea pigs on three different days at random as regards the site of injection Similar tests were given with the culture filtrates from Lind bII medium on three other groups of eight guinea pigs Each *preparation* was thus used in 24 tests

Injection and reading The technique for injection and reading of the reactions was the same as described previously (*Kim Magnusson & Bentzon* 1963) except

nsisted of 0 1 ml The reactions were read
aders The figures given in this paper are
› readers
of the culture filtrates was calculated as
nt on 1963) —The amount of tuberculin
ated from the formula

obs

$$\log D_{obs} = \frac{\overline{TR}_T \quad \overline{TR}_{ST}}{b_{ST}} + 1\,5$$

[1] The experimental work was performed in 1955

[2] The writers are grateful to *Dr K Tolderlund* and *Miss K Bunch Christensen* BCG Department Statens Seruminstitut for preparing and supplying the vaccine

where $\overline{TR}_T$ = average tuberculin reaction for the test tuberculin

$\overline{TR}_{ST}$ = average tuberculin reaction for the two standard doses and

$\bar{b}_{ST}$ = average slope of the tuberculin dose response curve = difference between reactions to the large and small standard doses The activity D (in tuberculin units per ml of culture filtrate) is calculated from $D = 200 \times 10 \times D_{01\,5}$ The 95 per cent confidence limits for the activity are $D \times 10^{\pm 2 s_E}$ [1]

RESULTS

Growth

On *Sauton medium* (Fig 1) there were only minor differences between the strains as regards growth rate and total bacterial crop For the majority of the strains the cultures were fully grown after four to five weeks The total crop was about 1300–1500 mg (180 ml culture medium), and slightly higher (1600–1700 mg) for E9656 only Strains T3505 and BCG showed different total bacterial crops in different flasks, 800 and 1300–1400 mg for T3505, 700 and 1300 mg for BCG In drawing the figures it has been presumed that there are two different forms of growth of these two strains The one (a) is characterized by a large total bacterial crop and usually a rapid acidification of the culture filtrate The other (b) is characterized by slower growth, smaller total bacterial crop and less change in pH, and is presumably due to sinking of the pellicle after a certain period of growth The same distinction between two forms of growth was made previously in a similar study (*Magnusson, Kim & Bentzon* 1963)

The strains made the medium more alkaline, pH 8 1–8 4 in the initial growth phase Later pH decreased to 5 0 or 5 5, except for the BCG strain where pH remained above 7 5 For E9656, Aoyama B, C and T3505 pH varied from 5 1 to 8 2 in the individual flasks at the final stage of growth

On *I and bII medium* (Fig 2) the differences between the strains as regards total bacterial crop were larger E5, C, U1921 and PN grew rapidly and the total bacterial crop was from 2000–2500 mg E9656, Aoyama B and T3505 grew more slowly and the total crop was smaller (1000 1500 mg) The growth of the BCG strain was very poor on this medium the pellicle sank after three weeks, and the total bacterial crop was about 800 mg

The rapidly growing strains E5, C, U1921 and PN at first made the medium alkaline (pH 8 2–8 8) and later acid (pH 5 6–6 1), and finally pH again increased to about 7 9, probably in relation to autolysis of the cultures (strains C, U1921 and PN)

The more slowly growing strains E9656, Aoyama B and T3505, also

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[1] Based on the average of results for the two readers an estimate of the standard deviation s = 1 74 was obtained The standard error is calculated as

$$s_E = \frac{s}{\bar{b}_{ST}} \times \sqrt{\frac{2}{24}} = \frac{0\ 359}{\bar{b}_{ST}}$$

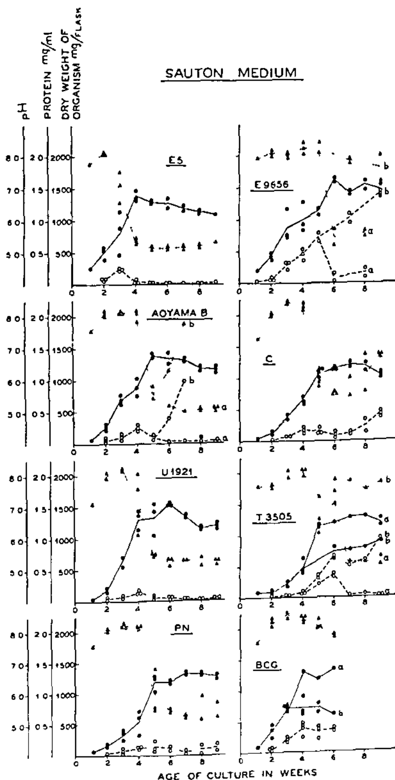


Fig 1

Dry weight of organism (●), pH (△) and protein content ( ) of culture filtrate of various strains of *Mycobacterium tuberculosis* cultured on Sauton medium in relation to age of culture For explanation of a and b curves, see text

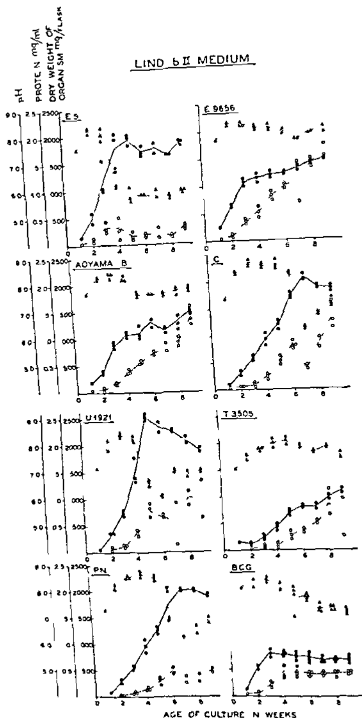


Fig 2

Dry weight of organism (●) pH (△) and protein content (○) of culture filtrate of vari us strains f *Mycobacterium tuberculosis* cultured on Lind bII medium in relati n to age of culture

made the medium alkaline (pH 8 1–8 4) at the beginning, but later the decrease in pH was only very slight and the pH remained above 7 5 Although growth of the BCG strain had apparently stopped after 3 weeks, there was a constant slight fall in pH after that time from 8 4 to 7 2 In a single flask pH was as low as 5 6

Protein Yield

For five strains, E9656, Aoyama B, C, T3505 and BCG, protein contents up to 0 4–1 4 mg/ml were measured on Sauton medium (Fig 1) The protein content remained below 0 3 mg/ml for the other strains

On Lind bII medium (Fig 2) the protein content of the culture filtrates was higher for all strains The highest values observed were 1 5 mg/ml (E9656), 0 9–1 4 mg/ml (Aoyama B, C, U1921 and T3505) and 0 4–0 6 mg/ml for the other three strains

Tuberculin Activity

The tuberculin activity of the culture filtrates of each strain, grown on Sauton medium, measured at the time when the tuberculoprotein content was at its maximum, is shown in Table 1 The strains are arranged in order of decreasing activity based on observations after 24 hours The age of the cultures varied from three to nine weeks The activity of the filtrate from strain E9656 (180 000 TU/ml) was 2–4 times larger than that of the other strains The filtrate from the C strain (115,000 TU ml) seemed to be approximately twice as active as the filtrate from the other six strains (50,000 75,000 TU/ml) The filtrate of E5 showed the smallest activity (50,000 TU/ml)

On Lind bII medium (Table 2) culture filtrates from strain C (240,000 TU/ml) and Aoyama B (210,000 TU/ml) were more active than the others The filtrate of BCG (85,000 TU/ml) had the smallest activity The age of the cultures used varied from six to nine weeks

Except for E9656, the filtrates on Lind bII medium were approximately twice as active as those from Sauton medium

The mean reactions decreased by 3 to 4 mm from 24 to 48 hours (Tables 1 and 2) However the results after 48 hours showed the same relationship as after 24 hours

There is no simple relationship between the tuberculin yield and the total bacterial crop of the various strains

Relationship between Protein Content and Tuberculin Activity

The logarithms of the amount of protein (in mg per ml) and the tuberculin activity (in TU per ml) of the culture filtrates have been calculated and the results are shown graphically in Fig 3 The points seem to be located on a line with slope 1 This means that the tuberculin activity

TABLE 1

Mean Size of Intradermal Reactions Read after 24 and 48 Hours (Erythema in mm) of BCG Vaccinated Guinea Pigs and Tuberculin Yields (in Tuberculin Units per ml) of Culture Filtrates of Eight Strains of Mycobacterium tuberculosis Cultured on Sauton Medium

Strain	Age of culture (weeks)	Dry weight of organisms§ (mg)	pH	Tuberculo-protein (mg ml)	24 hrs		48 hrs	
					Mean reaction* (mm)	Tuberculin yield† D (TU)	Mean reaction* (mm)	Tuberculin yield† D (TU)
I 9056	9	1400	8 0	1 41	15 9	180 000	12 7	220 000
C	9	1000	6 7	0 44	14 8	115 000	12 0	160 000
T 3505	8	800	7 5	0 57	13 8	75 000	10 2	70 000
Aoyama B	4	800	8 2	0 27	13 4	65 000	10 0	65 000
U 1321	4	1200	7 6	0 20	13 3	60 000	10 6	85 000
PN	5	1000	6 9	0 24	11 3	60 000	9 6	55 000
BCG	5	800	8 0	0 44	13 2	60 000	9 6	55 000
I 5	3	900	7 5	0 24	12 7	50 000	9 1	45 000

Mean reaction to standard solution containing 100 TU 16 1 mm 10 TU 10 6 mm (24 hrs)
" " 12 5 mm 10 TU 7 4 mm (48 hrs)

§ 180 ml medium
* Each figure is the mean of 24 reactions Dilution 1 200
† The 95 per cent limits of D are 0 73 × D and 1 36 × D

TABLE 2

Mean Size of Intradermal Reactions Read after 24 and 48 Hours (Erythema in mm) of BCG Vaccinated Guinea Pigs and Tuberculin Yields (in Tuberculin Units per ml) of Culture Filtrates of Eight Strains of Mycobacterium tuberculosis Cultured on I and bII Medium

Strain	Age of culture (weeks)	Dry weight of organisms§ (mg)	pH	Tuberculo protein (mg ml)	24 hrs		48 hrs	
					Mean reaction* (mm)	Tuberculin yield† D (TU)	Mean reaction* (mm)	Tuberculin yield† D (TU)
T 9656	8	1400	7 9	1 48	14 7	185 000	11 5	190 000
C	6	1800	8 4	0 91	15 4	240 000	11 9	230 000
T 3505	8	1000	7 8	1 13	13 9	135 000	10 0	100,000
Aoyama B	9	1500	7 9	1 39	15 0	210 000	11 8	220 000
L 1921	7	2300	7 0	0 94	14 4	165 000	11 2	165 000
PN	6	1500	8 1	0 55	13 9	135 000	10 5	120 000
BCG	9	700	7 3	0 49	12 6	85 000	9 5	80 000
I 5	9	1900	8 1	0 35	13 4	110 000	10 3	110 000

Mean reaction to standard solution containing 100 TU 14 9 mm, 10 TU 8 9 mm (24 hrs)
11 6 mm 10 TU 6 5 mm (48 hrs)

§ 180 ml medium
* Each figure is the mean of 24 reactions Dilution 1 200
† The 95 per cent limits of D are 0 73 × D and 1 36 × D

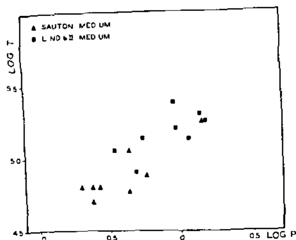


Fig 3
Tul erculin activity in relation to protein content of culture filtrate of *Mycobacterium tuberculosis*

Abscissa Logarithm of pr tein content in mg per ml culture filtrate
Ordinate Logarithm of tuberculin activity in TU per ml culture filtrate

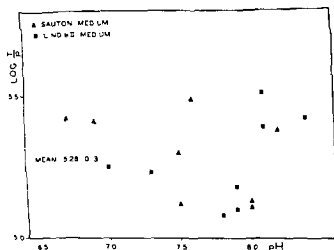


Fig 4
Tul erculin activity per mg of tul erculoprotein in relation to pH of culture filtrate of *Mycobacterium tuberculos s*

Abscissa pH of culture filtrate
Ordinate Logarithm of tuberculin activity in TU per mg of protein

per mg of protein is fairly constant—about 190 000 TU—for all the culture filtrates although it varies slightly within strains and is dif ferent on Sauton and L and b II medium for two of the strains (Aoyama B and U 1921) see also Fig 4 However there are no systematic differ ences between the two media in the tuberculin activity per mg of protein

and there is no clear-cut relationship between the pH of the culture filtrates and the tuberculin activity per mg of protein in this study (Fig 4) It should be remembered in this connection, however, that pH varies only from 6 7 to 8 4 in the culture filtrates under study

DISCUSSION

Previous studies have shown that there are differences between various strains of *M tuberculosis* as regards the yield and activity of the tuberculin obtained (*Seibert* 1934, 1937, *Lind* 1947, *Rzucidlo et al* 1952, *Svenkerud* 1955, *Szucs* 1955, *Takehara* 1957, *Magnusson & Bentzon* 1958, *Asami et al* 1961) *McIntosh & Konst* (1949) have made similar observations with different strains of *M bovis* On the other hand, *Paterson et al* (1958) report that two of the present strains, C and PN, behave similarly as regards tuberculin production In the present work, the tuberculin content was somewhat greater in the culture filtrates of strains E9656 and C than the other strains on Sauton medium Compared with Sauton medium, the yield of tuberculin was greater on Lind bII medium for all the strains, except one (E9656), and there was relatively less difference between the various strains BCG seemed to give a somewhat smaller yield than the other strains on Lind bII medium

In a previous study (*Magnusson, Kim & Bentzon* 1963) cultures of strain E9656 on Sauton medium were fully grown after 3 4 weeks At that time pH of the culture filtrate had decreased to 5 4, after which pH increased slightly again In all cultures more than two weeks old, however, pH was < 6 The protein content never exceeded 0 3 mg/ml In the present study cultures of E9656 on Sauton medium are fully-grown after 6 weeks, and even up to 9 weeks after the inoculation pH remains > 7 in some of the flasks The protein content is high (> 1 4 mg/ml) in these cultures

On Lind bII medium the growth of E9656 was very irregular in the first study (*Magnusson, Kim & Bentzon* 1963) Apparently the cultures were fully grown after 5 6 weeks, but there were large differences between flasks as regards total bacterial crop, pH and protein content up to 9 weeks of incubation In the present study the bacterial crop of E9656 cultured on Lind bII medium increased rapidly up to 3 weeks, and thereafter more slowly but quite regularly The pH remained >7 in all flasks except one The protein content was high and increased regularly with the age of the culture

Obviously the growth of strain E9656 has differed in the two studies It is believed that the ability of the pellicle to sink may be different in the two studies, see *Magnusson, Kim & Bentzon* (1963) *It is also believed* that the smaller tuberculin activity of the culture filtrate of the BCG strain on Lind bII medium is at least in part due to the sinking of the pellicle, se pg 359

The level of the tuberculin activity of the culture filtrates is slightly higher (50 000–240 000 TU/ml) in this study than in a previous one—25 000 200 000 TU/ml (*Kim Magnusson & Bentzon* 1963) This might be expected since culture filtrates with high protein contents (of varying ages) were selected for this study whereas 4 week old and 8 week old filtrates were used in the previous study The age of the cultures at the time the tuberculin activity was compared varied from 3 to 9 weeks A subsequent experiment gives the impression that the tuberculin activity of the culture filtrate is greatest when the concentration of tuberculoprotein is at its maximum It may therefore be assumed that the present study reflects actual differences between the strains as regards their ability to produce tuberculin The study shows that the tuberculin yield is twice as high from some strains as from others on Sauton medium Attention should therefore be paid to the yield when selecting strains for production of tuberculin However the results also indicate that the conditions necessary for obtaining the biggest yield (medium age of culture etc) vary from strain to strain

In the present study there is a closer relationship between the tuberculin activity and the protein content of the culture filtrates than in a previous study (*Kim Magnusson & Bentzon* 1963) This may be due—at least in part—to the variations in pH of the culture filtrates being less (from 6 7–8 4) in this study than in the previous one (5 4 8 5) In a subsequent study where pH of the culture filtrates of *M tuberculosis* has been changed within a wide range (1 8 12 3) by addition of acid or base a clear cut relationship between pH and the tuberculin activity per mg of protein has been found That no such relationship is seen in the present study (see Fig 4) may be due to the narrow range of variation of pH in this study

SUMMARY

[illegible]

str [illegible]

I [illegible] tuberculoprotein content of the culture filtrates were *determined* for the heat sterilized cultures The tuberculin activity of the culture filtrates was measured by intradermal tests on BCG vaccinated guinea pigs at the time when the tuberculoprotein content was at its *maximum*

On Sauton medium the tuberculin yield with one [illegible] the strains was [illegible] medium it was [illegible] In general the [illegible] pH was twice as large as on Sauton medium

No simple relationship was found between the tuberculin yield and the total bacterial crop of the various *strains* There was a close relationship between the protein content and the tuberculin activity of the

culture filtrates No clear-cut relationship could be found between the pH of the culture filtrates and the tuberculin activity per mg of protein

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FROM THE INSTITUTE OF FORENSIC MEDICINE, UNIVERSITY HOSPITAL, RIKSHOSPITALET, OSLO, NORWAY

A NEW SERUM TYPE SYSTEM IN MAN—THE Lp SYSTEM

By

KÅRE BERG

Received 25 iii 63

Since Smithies' discovery in 1955 of three different genetic types within the haptoglobins in normal human sera, several other serum type systems in man have been described (For references see. *Butler* 1961)

The distinction between genetic serum protein types depends partly on differences in electrophoretic mobility (the Hp-types, the transferrin types, and the Gc-types), partly on differences in the ability to inhibit agglutination of red blood cells coated with incomplete anti-D antibody by certain rheumatoid sera (the Gm-types, the Inv-types) Recently, also Ouchterlony's double diffusion technique in agar gel (*Ouchterlony* 1958) has been used to detect a serum type system (*Allison & Blumberg* 1961)

Allison & Blumberg (1961) described an isoprecipitin in the serum of a polytransfused patient In gel diffusion tests this precipitin distinguished between genetic types of a low density human serum β lipoprotein (*Blumberg, Dray & Robinson* 1962) This system was called the Ag system

(I

of

to ... specific antisera in animals were desired

Human β-lipoprotein was selected for such attempts because a genetic polymorphism within this protein already had been discovered by *Allison & Blumberg* (1961), and because isolation of relatively large amounts of the protein could be performed by simple means using chromatography on hydroxylapatite columns (*Tiselius, Hjerten &Levin* 1956 *Hjerten* 1959)

The hypothesis for the study was that animals, when given relatively large doses of an isolated protein from one single donor ...

... proper absorptions

The purpose of the present article is to describe a procedure by means of which a specific antibody was produced in rabbits This antibody

distinguished between different genetic types of human β lipoprotein which are not identical with earlier described types The purpose is furthermore to present evidence for the heredity of this serum type system, and to discuss the possible use of the procedure described in detecting new genetic systems in man

The antigen of the serum type system to be described human β lipoprotein, is identical to the α-2 lipoprotein demonstrated with immuno electrophoresis (*Crowle* 1961, p 96), and almost synonymous with the 'low density lipoprotein' in ultracentrifugal peparation (*Cramer* 1962)

MATERIALS AND METHODS

Human Sera

A panel of 20 normal human sera from laboratory personnel was used for isolation of β lipoprotein absorption experiments and specific absorption controls of rabbit antisera

Sera from blood donors (kindly provided by the Blood Bank Rikshospitalet Oslo) and from unrelated adults involved in paternity cases submitted to the Institute of Forensic Medicine were used for calculation of gene frequencies

A family material consisting of 23 families with 65 children was kindly placed at my disposal by Dr Jan Mohr Institute of Medical Genetics University of Oslo (These are the first families studied of a larger family material see *Berg & Mohr* 1963)

All blood samples with the exception of those from the family material were obtained by venepuncture The blood was allowed to clot at 37° C for 2 hours and kept overnight at 4° C Serum was then pipetted off centrifuged free of corpuscular elements and stored at —25° C until used From the persons of the family material capillary blood was drained from the ear lobe and treated in the same way

Rabbits

Albino rabbits weighing approximately 3 5 kg were used for the immunization procedures

Polyvalent Rabbit Anti Human Serum Antiserum

The serum (K 467) was obtained by immunization of a rabbit with pooled whole human serum

Sheep Anti Rabbit Serum Antiserum

This was kindly provided by *Dr J Ulstrup* Oslo City Hospital Oslo

Specific Rabbit Anti Human β Lipoprotein Antiserum

Anti α 2 Lipoproteinserum Op Nr 320 D from *Behringwerke AG* Marburg was used

Dialysis Bags

Cellophane casings from *Visking Co* Chicago Ill

Hydroxylapatite

The material for column chromatography was prepared according to *Tiselius Hjerten & Levin* (1956)

Isolation of Human β Lipoprotein

The method described by *Hjerten* (1959) was used see under Experiments and Results

Concentration of Chromatographic Fractions

The method described by *Kohn* (1959), using Polyethylene glycol Hoechst 20 000, was applied

Petri Dishes

For gel diffusion tests *ANUMBRA* dishes 7 cm diameter were used

Agar

2 per cent Difco Bacto agar, washed according to *Hirschfeld* (1960) was stored in 12 ml portions at 4° C until used

Agar Double Diffusion Tests by Ouchterlony's Technique

The petri dishes were thoroughly cleaned In each dish was poured 12 ml of molten 2 per cent agar and an equal amount of a solution thus prepared that the resulting 1 per cent agar in the dishes contained 0 85 per cent NaCl, 1/10 volume 1 15 M sodium phosphate buffer pH 7 0, and 1/10 000 (weight/volume) merthiolate With a cutter six peripheral wells were cut around one central well, and the bottom of each well was sealed with a thin layer of molten agar The diameter of each well was 4 mm and the distances between the circumferences of neighbouring wells were in all cases 5 mm The rabbit immune sera were placed in the central wells and the human sera in the peripheral wells The dishes were kept in a moist chamber at 37° C. Readings of precipitates were made against a dark background by oblique illumination from below, after 1, 2, 3 and 7 days The reading after 2 days was regarded as decisive since no changes occurred after that time

Immunoelectrophoresis

This was performed according to *Grabar & Williams* (1953) with the micromodification described by *Scheidegger* (1955) For purity test of the β lipoprotein fraction a modified immunoelectrophoretic technique proposed by *Heremans* (1960) was also applied

Staining

Staining of the immunoelectrophoretic and Ouchterlony preparations with lipoid and protein stains was performed according to *Grabar & Burtin (1960), using Oil Red O* as a lipoid stain and Amido Black 10 B as a protein stain both from *G T Gurr Ltd* London

Photographic Registrations

Photographic registrations of gel diffusion and immunoelectrophoretic tests were made with a Leica 35 mm camera on Agepe Agfa film

EXPERIMENTS AND RESULTS

Preparation of Human Serum β-Lipoprotein

8 5 ml of fresh, undialysed normal human serum was fractionated on a hydroxylapatite column (diameter 13 mm, length 150 mm) with a stepwise elution technique Three different phosphate buffers, all with a pH of 6 8, were used for elution, increasing the molarity successively in the order 0 10, 0 25, and 0 65 M, according to *Hjertén* (1959) The eluate was collected in 5 ml fractions and the optical density of each fraction measured at 280 mμ in a Beckman DU spectrophotometer The five fractions representing each peak of the chromatogram (see Fig 1) were concentrated by dialysis against polyethyleneglycol to approxima-

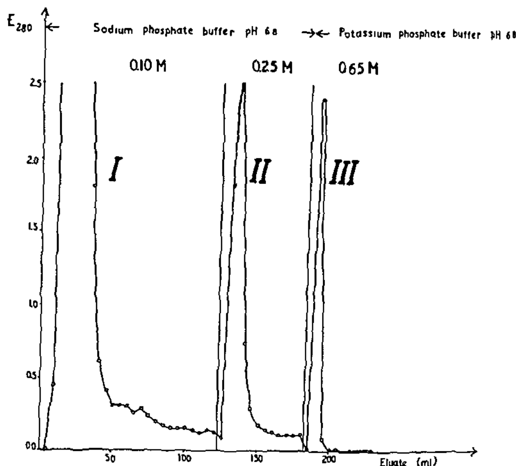


Fig 1

Chromatography of 8.5 ml whole human serum on a hydroxylapatite column (diameter 13 mm length 150 mm) for preparation of β lipoprotein

tely 1/5 of their original volume, and examined qualitatively by immunoelectrophoresis

Peak I and peak II contained together all other serum proteins and no demonstrable β lipoprotein, while peak III consisted exclusively of this protein The purity of the β lipoprotein fraction was verified in gel diffusion tests against polyvalent rabbit-anti-human-serum-antiserum, and by a modified immunoelectrophoretic technique proposed by Heremans (*Heremans* 1960, p 40), see Fig 2

The β-lipoprotein fraction reacted positively in gel diffusion test against specific anti α-2-lipoprotein serum from Behringswerke

All precipitates formed by this protein stained brightly red by the lipoid stain Oil Red O

The β-lipoprotein migrated down the column as a narrow orange-coloured band during the elution with 0.65 M potassium phosphate buffer, thus offering the possibility to obtain the greater part of it in one batch by visual control

Spectrophotometry showed that the greatest concentration of the



Fig 2

proteins

protein was obtained in this manner This batch was dialysed for 24 hours against four changes of one litre of a 0 85 per cent saline solution at 4° C After dialysis the average volume was 5 8 ml and the average optical density E ₀ — 2 355 All β lipoprotein batches from the different separation experiments were adjusted to give this extinction This final product was used as a standard solution for immunization

Immunization Experiments

Three rabbits were immunized with the standard β lipoprotein solution prepared from the serum of one single individual chosen at random from the panel Two ml of the solution were injected intravenously in the marginal vein of the ear daily for the first three days of four successive weeks The total dose was thus 24 ml for each rabbit No adjuvants were used

Blood was collected from the marginal vein of the rabbit s ear seven days after the last injection incubated for 2 hours at 37° C and kept overnight at 4 C Serum was then pipetted off centrifuged free of corpuscular elements and stored at 25° C until used

Testing of Antiserum

For control sera from all rabbits collected before the immunization procedure started were tested against whole normal human serum and all fractions obtained by chromatography in gel diffusion tests No precipitins could be detected in the sera of the rabbits before immunization

After the immunization course serum from all three rabbits gave precipitates with all normal human sera and with all isolated β lipoprotein fractions with the Ouchterlony technique

By immunoelectrophoresis it was demonstrated that the rabbit im

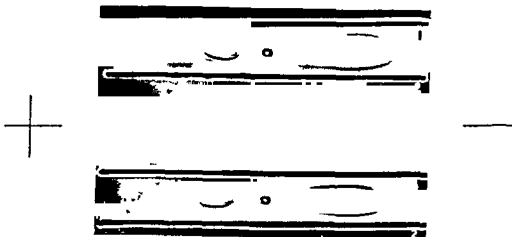


Fig 3

Identification of antibodies of rabbit serum Rabbit immune serum was placed in the two small circular wells and submitted to electrophoresis Afterwards the longitudinal groove 1 was filled with β lipoprotein fraction the grooves 2 with sheep anti rabbit antiserum and groove 3 with whole human serum The precipitate pattern shows that the rabbit antibodies directed against human β lipoprotein and whole human serum is located in the gamma globulin region

mune sera gave a heavy precipitate with β-lipoprotein but faint bands corresponding to albumin, gammaglobulin and β-2-M-globulin were also present The probable reason for this polyvalency of the rabbit immune sera is that the β-lipoprotein solution used for immunization was contaminated with traces of other proteins, in spite of the fact that these could not be demonstrated by the methods used for testing its purity

To identify the protein fraction of the rabbit immune serum containing the precipitating antibodies, a special immunoelectrophoretic procedure was applied, as shown in Fig 3 The rabbit immune serum was subjected to electrophoresis and afterwards to diffusion against normal human serum on one side, and a sheep anti rabbit serum-antiserum on the other These experiments showed that the precipitating antibodies of the rabbit immune sera belonged to the γ-globulins

In all experiments described above, serum from the 3 rabbits gave identical results

Absorption Experiments

Whole human serum and isolated β-lipoprotein from the panel donors were used as absorption materials Undiluted rabbit immune serum and

absorption material were mixed in different proportions in glass tubes The tubes were incubated one hour at 37° C tilted 10 15 times during the incubation period and kept overnight at 4° C The mixtures were then centrifuged 10 minutes at 1600 G the supernatant was pipetted off the sedimented precipitate and stored at —25° C until used The absorbed antisera were tested in Ouchterlony dishes against whole serum and isolated β lipoprotein fractions from the panel The reactions were read after 48 hours and visible precipitates registrated as positive reactions no precipitate as negative reactions

These experiments demonstrated a distinct difference between two types of donors of absorption material which for reasons later explained were called positive and negative reactors

When the immune sera were absorbed with serum from a negative reactor in a certain range of proportions they no longer reacted positively with all but only with some normal human sera If however serum from a positive reactor was used for absorption the immune sera lost their precipitating ability at a relatively low concentration of absorption material and against all human sera at the same absorption ratio The experiments thus indicated that rabbit immune sera absorbed with serum from a negative reactor in certain proportions were able to distinguish between two types of normal human sera It was assumed that the immune sera thus absorbed had become specific for a factor present in some but not all human sera Further experiments showed that 7 of the 20 panel sera contained this factor (positive reactors) while 13 did not (negative reactors) Serum from any of the latter could be used as absorption material to produce specific antisera As expected the arbitrarily chosen donor of the β lipoprotein used for immunization of the three rabbits was found to be a positive reactor Typical reaction patterns between the three rabbit immune sera at different degrees of absorption and serum from positive and negative reactors are shown in Table 1 The table also includes the reactions when the immune sera were absorbed with serum from a positive reactor

For practical purposes only the rabbit immune sera (K 1 and K 3) remaining specific at a wide range of absorption ratios were used as typing reagents In all further experiments serum K 1 was used absorbed 2 3 and serum K 3 absorbed 2 5 with serum from a negative reactor These two antisera gave the same reactions with more than 300 normal human sera which demonstrated that their specificities were identical

The reproducibility of the reactions of individual human sera was demonstrated in blood samples taken at different intervals during a period of more than one year The reagibility of human sera remained unchanged when stored at —25° C for several months but after ½ 1 year positive reactions seemed to become weaker

Isolated β lipoprotein gave the same reactions with the type specific antisera as whole serum from the same donor The specific antisera

TABLE 1

Reaction Pattern by Double Diffusion in Agar Gel between Absorbed Rabbit Anti-Human β Lipoprotein-Antiserum and Serum from Positive and Negative Human Reactors (+ = Visible Precipitate, — = no Visible Precipitate)

Immune serum from rabbit	Normal human serum from	Ratio (volumes) Rabbit immune serum serum from negative human reactor														Ratio (volumes) Rabbit immune serum serum from positive reactor					Controls Unabsorbed rabbit immune serum
		2:1	2:2	2:3	2:4	2:5	2:6	2:7	2:8	2:10	2:12	2:14	2:16	2:18	2:20	2:1	2:2	2:3	2:4	2:6	
K 1	Pos reactor	+	+	+	+	+	+	+	+	+	(+)	—	—	—	—	—	—	—	—	—	+
	Neg reactor	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+
K 2	Pos reactor	+		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+
	Neg reactor			—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+
K 3	Pos reactor	+	+	+	+	+	+	+	+	+	+	+	+	(+)	—	(+)	(+)	—	—	—	+
	Neg reactor	+	(+)	—		—	—	—	—	—	—	—	—	—	—	(+)	(+)	—	—	—	+

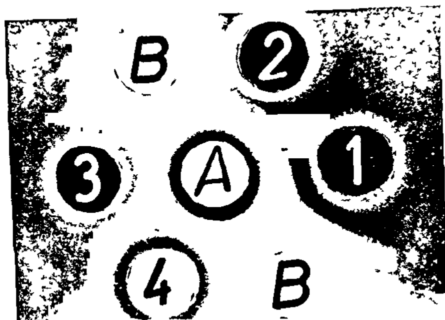


Fig 4

Lipid stained Ouchterlony dish showing reaction pattern between specific absorbed rabbit immune serum and β lipoproteins and whole human serum from positive and negative reactors

The reagents were: A Rabbit immune serum absorbed specific with whole human serum from a negative reactor.—B Rabbit immune serum absorbed specific with isolated human β lipoprotein from a negative reactor.—1 Whole human serum from a positive reactor. 2 Isolated β lipoprotein from a positive reactor.—3 Isolated *β lipoprotein from a negative reactor.*—4 Whole serum from a negative reactor

gave no reactions with other serum fractions from the chromatographic experiments. Rabbit immune sera could also be absorbed specific with isolated β lipoprotein from negative reactors, see Fig. 4.

It was thus evident that specific rabbit immune sera revealed a type specificity of human β lipoprotein.

The Relation to the Ag System

Preliminarily it seemed probable that the revealed type specificity of human serum β lipoprotein might constitute a hereditary system.

The Ag system (*Allison & Blumberg* 1961, *Blumberg, Dray & Robinson* 1962) also refers to a property of human β lipoprotein and in order to find whether any relationship exists between the Ag system and the type specificity observed in the present study a comparative investigation was undertaken. Twenty three normal human sera already tested with specific absorbed rabbit immune serum were kindly tested with anti Ag (a) serum (C de B) by Dr. B. S. Blumberg of the National Institutes of Health, Bethesda, Maryland, U.S.A. No apparent relation

ship was found between the Ag system as defined by this antiserum, and the factor detected by means of the specific absorbed rabbit immune sera (se also *Berg & Mohr* 1963).

Nomenclature

When it was evident that the property revealed by the specific rabbit immune sera was not related to the Ag system, a new nomenclature was regarded necessary. The factor present in the serum of positive human reactors was called the Lp(a) factor, where Lp is an abbreviation for Lipo-protein. In the following, individuals possessing this factor are said to be of type Lp(a+), and those lacking the factor of type Lp(a—). Accordingly the presumed hereditary system was called the Lp system, and the specific antibody present in rabbit immune sera anti Lp(a).

Frequency of the Types Lp(a+) and Lp(a—)

Sera from 314 healthy, unrelated adult individuals were tested against the rabbit immune sera prepared as described above. Of the 314 human sera, 107 (34.08 per cent) were of type Lp(a+), and 207 (65.92 per cent) of type Lp(a—).

Preliminary Genetic Studies

It was supposed that the β-lipoprotein types form a system which is determined by a pair of genes, provisionally called Lp^a and Lp^x. According to this assumption, the gene Lp^a expresses itself whether present in single or double dose, and the relation between pheno- and genotypes is as shown in Table 2.

From the frequencies in the preceding paragraph, the gene frequencies were calculated, see Table 3.

In order to test the theory, a genetic study was undertaken. For this purpose, sera from members of 23 families were tested. The type results are summarized in Table 4.

TABLE 2

Relation between Pheno- and Genotypes in the Lp System

Phenotype	Possible genotypes
Lp(a+)	Lp^a/Lp^a or Lp^a/Lp^x
Lp(a—)	Lp^x/Lp^x

TABLE 3

Gene Frequencies of the Lp System

Frequency of gene Lp^x	$\sqrt{(Lp(a-))}$	= 0.8119
Frequency of gene Lp^a	$1 - Lp^x$	= 0.1881

(Lp(a—)) denotes the frequency of phenotype Lp(a—).

TABLE 4

Family Material Investigated with Anti Lp(a) Antiserum from Rabbits

Parents			Children				
Matings	Obs	Exp	Total	Lp (a+)		Lp (a−)	
				Obs	Exp	Obs	Exp
Lp(a+) × Lp(a+)	4	2.7	13	9	10.4	4	2.6
Lp(a+) × Lp(a−)	9	10.3	27	19	14.9	8	12.1
Lp(a−) × Lp(a−)	10	10.0	25	0	0	25	25

In this table, the expected results are calculated from the gene frequencies given above. No exceptions from the outlined theory of inheritance were observed, and the observed numbers of children within each category of mating fit reasonably well with the expected (see also *Berg & Mohr* 1963).

This study therefore verifies that the Lp types are genetically determined.

DISCUSSION

By the combination of a certain immunization program in rabbits and a specific absorption procedure, antisera have been produced which reveal a hitherto unknown inheritable factor of human serum β lipo protein. The underlying principle depending upon the ability of the animal to distinguish between individual antigenic differences in man, is by no means new. This ability has been practically employed ever since *Landsteiner & Levine* (1927) demonstrated the M and N types of human erythrocytes. Specific antiserum against an atypical human haptoglobin has been produced by *Alg, Brinker, Deicher, Hartmann & Nix* (1962) by absorption of horse-anti-human-serum-antisera with normal human haptoglobin, type Hp 2–1. Successful attempts to produce heteroantisera against normal individual serum factors have not yet been reported, however.

Blumberg, Dray & Robinson (1962) have reported attempts to produce antisera antithetic to anti Ag(a) by immunizing chimpanzees with human serum of type Ag(a—). Whole human serum together with Freund's adjuvant was used for the procedure, but no specific antibodies could be demonstrated in the antisera.

The experiments described in the present paper are based on the following points, one or more of which may be essential for the positive results.

1. A purified protein fraction was used for immunization instead of whole human serum.
2. Each rabbit was immunized with a serum protein fraction from one single individual.

3 The antigen was given intravenously by repeated injections without the use of adjuvants

4 The immune sera thus obtained were submitted to a specific absorption procedure

It is at present not possible to state whether any of the steps mentioned above are necessary for the results or may be substituted by alternative procedures It seems logical, however, to believe that the selection of one single donor is essential for the production of antibodies against yet unknown individual specific factors Experiments in order to define the optimal conditions for the production of such antibodies are in progress

The experiments described seem to prove that the Lp(a) factor is a characteristic of the human serum β lipoprotein They further suggest that more than one antigenic factor are present in this protein, of which one or more are common to all humans The antibodies against the unspecific factor or factors are believed to be removed during the absorption procedure, thus revealing the type specificity of the antiserum

There is at present no reason to believe that these principles should only be valid for the β-lipoprotein of human serum, although it yet remains to be demonstrated that other serum type systems can be established in this way

It may even be suggested that individual specific types of tissue antigens can be found by means of related procedures

It is possible, that in analogy with the conditions in the MN system, the gene *Lp*^{a} also expresses itself in a β-lipoprotein factor with hetero-antigenic properties

Attempts to produce rabbit immune sera antithetic to anti-Lp(a) have been undertaken, using human β-lipoprotein from Lp(a—) individuals for the immunization procedure So far these attempts have been unsuccessful The rabbits produced strong antibodies directed against human β-lipoprotein, but till now it has not been possible to make the immune sera type specific by absorption It must be remembered, however, that one can only expect successful specific absorption when the serum from an individual of genotype *Lp*^{a}/*Lp*^{a} is used as absorption material At present, no differentiation can be made between genotypes *Lp*^{a}/*Lp*^{a} and *Lp*^{a}/*Lp*^{a}, and as the latter genotype is rather rare, numerous experiments must be done before one can be sure that the correct absorption material has been used A possibility would be to test the positive reactor of Lp(a+) × Lp(a—) matings with many children, all of type Lp(a+)

If the serum of an individual of genotype *Lp*^{a} *Lp*^{a} was available, one could also try to make the rabbits tolerant for this type β-lipoprotein by immunizing newborn animals Later the injection of β-lipoprotein from individuals of type Lp(a—) would follow The possibility to use animals other than rabbits for the immunization procedure must also be considered

SUMMARY AND CONCLUSIONS

An immunization procedure consisting of intravenous injections of isolated human serum β-lipoprotein from one single individual followed by a specific absorption program of the immune sera, has led to the production of specific rabbit immune sera distinguishing between individual types of human serum β-lipoprotein

Genetic studies have shown that these β-lipoprotein types constitute a hereditary system different from earlier described serum type systems Comparative studies have shown that the system is also different from the Ag system which also refers to a property of human β-lipoprotein The new system is provisionally called the Lp system

The possible application of the procedure for production of specific hetero antibodies in the search for still unknown genetic systems of human serum proteins and even tissue antigens is discussed

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FROM THE TUBERCULOSIS AND STATISTICAL DEPARTMENTS STATENS SERUMINSTITUT, AMAGER BOULEVARD 80, COPENHAGEN S DENMARK

ACQUIRED RESISTANCE TO TUBERCULOSIS INDUCED IN RED MICE (CLETHRIONOMYS G. GLAREOLUS SCHREB.) BY VACCINATION WITH LIVING BCG

2 *Determination of Dosage Range for Challenge*

By

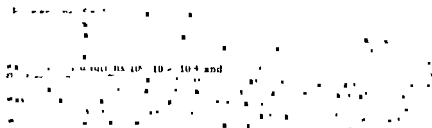
ANDR JESPERSEN and M WEIS BENTZON

Received 26 iii 63

In order to find a method for evaluation of the acquired resistance induced by a BCG vaccine, using red mice as experimental animals, a study was carried out previously to examine the effect of various doses of BCG on a challenge with a relatively small dose of bovine tubercle bacilli (5) Doses from 10 ⁻⁴ to 10 ⁻⁹ mg of BCG caused prolongation of the survival time of the animals after challenge as compared to non-vaccinated animals The interval between vaccination and challenge was seven weeks

The aim of the present work was to determine the dosage range for challenge which can be used to demonstrate acquired resistance in BCG vaccinated red mice Both the dose of vaccine and the challenge dose were varied within wide limits Groups of red mice were vaccinated with doses of BCG ranging from 1–2 units to 1.2×10^{6} *units and* subsequently challenged with doses of tubercle bacilli ranging from 30 to 30×10^{6}

EXPERIMENTAL



This work was carried out with the aid of a grant from "P Carl Petersens Fond

MATERIAL AND METHODS

Experimental animals The mice which were bred in the animal quarters of Statens Seruminstitut were 2½ to 5½ months old at the commencement of the experiment. A total of 500 mice were used. When placing the mice in cages alternate cages were given consecutive numbers from 1 to 125 and the females distributed first at random with two animals per cage. The males were then allotted to the remaining cages according to the same system. Each experimental group contained 10 females and 10 males. The cages which measure 17 × 24 × 17 cm are of glass with bottom and corners of zinc and are fitted with perforated iron lids.

Diet The mice were given a daily diet of whole oats and diluted skimmed milk with mineral salts and vitamins added as well as water ad lib from water bottles. Three times a week the diet was supplemented with greenstuff, in the form of carrots, lettuce and spinach according to the season.

Vaccination with BCG vaccine The vaccine was a fresh batch (Lot No 1304) prepared in the BCG Department of the Institute containing 40 mg of semi dried BCG per ml.

Suspensions of 10^{-1}, 10^{-2} ... 10^{-7} mg BCG in 0.2 ml were prepared with 0.1 per cent bovine albumin as diluent.

Before and after vaccination, which process took about three hours, Lowenstein Jensen medium was inoculated from the two lowest dilutions. Culture of 0.1 ml (half the inoculum per mouse) gave the following colony counts

Before vaccination

1/2 10^{-6} mg BCG	9 7 6 6 8–10 8 8 11 cont	Average 8.1 colonies
1/2 10^{-7} mg BCG	1 0 0 0 1–1–1–1 1 0	Average 0.6 colonies

After vaccination

1/2 10^{-6} mg BCG	5 5 5 10 cont –3 4–1 5 0	Average 4.2 colonies
1/2 10^{-7} mg BCG	1–0 0 0 1 1 0 0 0 1	Average 0.4 colonies

Colony counting showed that before the vaccination the 10^{-6} suspension contained an average of 16.2 bacterial units and the 10^{-7} suspension 1.2 units per 0.2 ml. After vaccination the counts were 33–50 per cent lower.

Vaccination was carried out in the following order 10^{-7} mg, 10^{-6} mg ... 10^{-1} mg. Within each dose the animals were vaccinated cagewise at random. The cages were given 40 random numbers and then placed in numerical order and the animals vaccinated according to the consecutive cage numbers. The control animals were injected with 0.2 ml of bovine albumin. The vaccination was made subcutaneously *in the groin of the right leg with 0.2 ml of suspension.* The vaccine suspensions were protected against daylight during preparation and use.

Vaccination period The interval between vaccination and challenge was three months.

Challenge Infection of the 480 animals was carried out by intravenous injection *of 0.2 ml of bacterial suspension into a tail vein. The process took two days,* on the first day dilutions 10^{-6} and 10^{-4} being used and on the second 10^{-2} and 10^{0}. The animals were injected cagewise at random. The strain used was a virulent bovine strain I 6884 B. A 13 day old culture grown in Dubos fluid medium containing Tween 80 was subjected to ultrasonics for ten minutes at an intensity of 0.56 watts per cm² *(12) and then diluted to 10^{0} with diluted Sauton medium in the ratio 1:9.* The suspensions 10^{0} and 10^{-2} were placed in the refrigerator until the next day. The number of viable bacterial units in the suspension was determined on each of the two days by culture on Lowenstein Jensen medium, 0.1 ml being inoculated on to 10 tubes for each of the dilutions 10^{-4}, 10^{-5} and 10^{-6}. Culture was carried out immediately before and after the challenge process *which took about 3–3½ hours* per day. The bacterial suspensions were protected against daylight during preparation and use.

By means of differential counting of the clumps in a Ziehl Neelsen stained slide from a suitable dilution of the ultrasonic treated bacterial suspension the number of bacteria per unit was calculated to be *1.8. Colony counts from the first and second*

day showed that the 10⁴ suspension contained 6 3 units and 11 0 units per 0 1 ml respectively, i e an average of 8 7 units Thus the dose per animal infected with that suspension contained about 17 bacterial units or about 30 bacteria The colony counts before and after challenge showed a slight decrease on both days

The mice were observed until they died spontaneously The degree of tuberculous infection in the mouse was determined on the basis of autopsy findings and the number of bacilli in smears from liver spleen and lungs If the organs showed no definite macroscopic signs of tuberculosis culture was carried out on Löwenstein Jensen medium The degree of infection was classified as in previous experiments as Tub O to Tub V

The survival times after day of challenge for the mice with moderate or severe generalized tuberculosis (Tub IV or V) were used as indication of the acquired resistance

In the interval between vaccination and challenge 63 mice died The deaths were distributed evenly over the various groups of animals and were not attributable to the vaccination Though red mice are extremely sensitive to infection with virulent bovine tubercle bacilli they can tolerate enormous doses of BCG even when injected intraperitoneally or intravenously

TABLE 1

Mortality among Experimental Animals in the Interval between Vaccination and Challenge

	Survivors	Deaths
	479	
17 31/12 1959		27
	452	
1 10/1 1960		12
	440	
11 20/1		4
	436	
21 30 1		5
	431	
31 1 9 2		4
	427	
10 19 2		2
	425	
20 29/2		2
	423	
1 10 3		4
	419	
11 20 3		3
	416	
		63

Table 1 gives the mortality in the vaccination period at intervals of ten days while Fig 1 shows the survival times in diagrammatic form The cause of the many deaths which accumulated between Christmas and the New Year when the care of the mice was in the hands of other than the usual staff was probably that for a day or two the mice did not get enough fluid From January 15 water was given ad lib from drinking flasks During the last five ten-day periods before challenge there

was an almost constant mortality of 3 out of about 420 *i e* 0 7 per cent which is quite usual in experiments of this kind Two of the 20 mice which were neither vaccinated nor infected died before the other animals were challenged After that time one died within the first 100 days and five within the next 100 days while eight lived more than 600 days

RESULTS

Table 2a b, c, d, gives the survival time and grade of tuberculosis for each animal, and furthermore the survival times are shown diagrammatically in Fig 2a, b, c, d There was no difference in the survival times of the male and female animals, and thus these are taken together As in previous experiments, it has been found that the reci

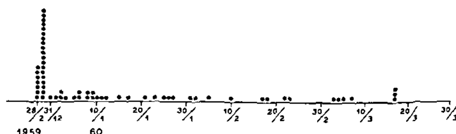


Fig 1

Occurrence of deaths in the vaccination/challenge interval

TABLE 2 a

Survival Times and Degree of Tuberculosis in BCG Vaccinated Red Mice Infected with Different Doses of Bovine Tubercle Bacilli Challenge Dose 10^{0} 30×10^{6} tubercle bacilli

Non vaccinated controls		Vaccination dose									
		10^{-7} mg BCG		10^{-6} mg BCG		10^{-5} mg BCG		10^{-3} mg BCG		10^{-1} mg BCG	
10	IV	10	V	6	IV	7	IV	9	V	3	I
10	V	10	IV	8	IV	8	IV	9	IV	8	IV
12	V	10	V	8	IV	8	IV	10	IV	9	V
12	V	12	V	9	IV	9	IV	10	V	9	V
12	V	12	V	10	V	9	IV	10	V	10	V
13	V	12	IV	10	IV	9	IV	10	V	10	V
13	V	12	V	10	IV	9	IV	10	IV	11	V
13	V	13	IV	10	V	10	V	10	V	11	V
13	IV	13	V	10	V	10	IV	11	V	11	V
13	V	13	V	11	V	11	IV	11	V	12	V
14	V	14	V	11	IV	14	V	11	V	12	V
14	V	14	V	11	V	14	V	12	V	12	V
14	V	14	V	12	V	15	V	13	IV	12	V
16	V	14	V	12	V	16	IV	13	V	14	V
16	V	14	V	14	V	16	V	17	V	15	V
16	V	14	V	14	V	17	V	17	V	17	V
23	V	16	V	16	V			17	V	*	
		16	V	*				19	V		
*				*							
**		*		*				*			

The table shows the survival times of the animals in days after challenge
The Roman figures indicate the degree of tuberculosis at time of death
* Died during vaccination/challenge interval
** Challenge inoculation unsuccessful

TABLE 2 b

Survival Times and Degree of Tuberculosis in BCG Vaccinated Red Mice Infected with Different Doses of Bovine Tubercle Bacilli Challenge Dose $10^{-?}$ 30×10^{1} tubercle bacilli

Non vaccinated controls		Vaccination dose									
		10^{-7} mg BCG		10^{-6} mg BCG		10^{-5} mg BCG		10^{-3} mg BCG		10^{-1} mg BCG	
17	IV	19	IV	21	V	24	IV	10	decomp	50	V
19	IV	19	IV	29	V	41	III	57	V	56	IV
19	IV	21	IV	46	V	51	III	60	IV	58	V
21	IV	21	IV	67	V	52	V	63	IV	65	V
22	V	22	IV	69	V	53	V	68	IV	69	IV
22	V	24	V	70	V	53	IV	71	V	71	V
24	IV	28	V	73	IV	57	V	72	V	77	V
24	IV	28	V	76	V	61	IV	73	V	80	V
24	V	30	V	76	V	63	V	77	V	85	V
25	V	31	V	77	V	64	IV	78	IV	90	V
26	V	41	V	79	V	71	IV	81	V	94	IV
27	IV	41	V	80	IV	71	IV	81	V	95	V
27	V	46	V	96	V	74	V	83	V	95	IV
36	V	49	IV	98	V	80	V	86	V	96	V
42	V	50	IV	102	V	81	IV	94	V	97	V
54	V	53	V	104	V	84	V	104	V	103	V
		61	V	108	V	87	V	104	V	114	V
		81	V	110	V	91	IV	130	V	136	V
*		*		132	V	91	V	*		167	V
		*		*		95	V	*		*	

The table shows the survival times of the animals in days after challenge
The Roman figures indicate the degree of tuberculosis at time of death
* Died during vaccination challenge interval

procal value of the survival times could be regarded as normally distributed A statistical evaluation of the survival times for the various infection doses is given in Table 3 The arithmetic means of the reciprocal survival times (y), the standard deviation (s_y) and the median survival time in days, are shown for each separate group

Challenge dose 10^{0} (30×10 tubercle bacilli) The effect of the vaccination manifested itself here as a reduction in the survival time The median survival times for the groups vaccinated with 10^{-6}, 10^{-5} and 10^{-1} mg BCG were significantly shorter than those for the 10^{-7} and control groups In the latter groups the $s_y = 0.0126$ The standard error of the difference between two y values (n = about 17) is 0.0043 The median survival time for the 10^{-3} group deviated significantly from the control group but not from the 10^{-7} group It is of interest that the standard deviation for groups 10^{-6} to 10^{-1} as a whole was greater than for the 10^{-7} and control groups In all groups the animals which lived longest died on the 16th 17th day (two animals died on the 19th and 23rd day), but in the 10^{-6} to 10^{-1} groups a number of deaths occurred on the 8th and 9th day This was not seen in the other groups

Challenge dose $10^{-?}$ (30×10^{1} tubercle bacilli) Here the vaccination induced a definite increase in the resistance of the animals All the

TABLE 2 c

Survival Times and Degree of Tuberculosis in BCG Vaccinated Red Mice Infected with Different Doses of Bovine Tubercle Bacilli Challenge Dose 10^{-4} 3000 tubercle bacilli

Non vaccinated controls		Vaccination dose 10^{-7} mg BCG		10^{-6} mg BCG		10^{-5} mg BCG		10^{-3} mg BCG		10^{-1} mg BCG	
2	0	29	0	69	IV	3	I	78	IV	96	III
20	III	50	III	101	V	49	I	101	IV	97	I
38	III	56	IV	104	IV	89	III	122	IV	107	I
69	IV	57	IV	125	IV	103	III	138	III	131	IV
76	*V*	*58*	*IV*	*131*	*V*	*109*	*IV*	*142*	*V*	*137*	*III*
80	V	58	V	134	IV	113	V	172	V	150	V
81	V	63	IV	167	V	126	IV	173	I	156	IV
92	V	69	IV	173	V	127	V	200	III	159	V
93	IV	81	V	188	V	153	V	200	V	164	V
98	V	85	IV	193	V	158	IV	223	V	164	V
98	V	87	V	195	V	160	V	239	V	183	V
116	V	91	V	205	IV	161	V	276	V	210	V
133	V	92	IV	209	V	183	IV	317	V	243	V
137	V	95	V	210	V	230	V	383	IV	252	V
138	V	112	V	253	V	237	V	387	V	254	V
*		135	V	274	V	301	V	446	V	270	V
*		144	V	*		322	V	449	V	285	V
*		192	V	*		*		*		470	V
*		224	V	*		*		*		*	
*		*		*		*		*		*	

The table shows the survival times of the animals in days after challenge
The Roman figures indicate the degree of tuberculosis at time of death
* Died during vaccination/challenge interval

mice vaccinated with 10^{-6} to 10^{-1} mg of BCG had a prolonged survival time The standard deviation s_y for the control and 10^{-7} groups is 0 0120 or almost the same as with challenge dose 10^{0}, i e the standard error is 0 0042 The median survival times varied from 63 to 82 days as against 25 days in the control group In the 10^{-6} and 10^{-5} groups the survival times were shorter and the standard deviation somewhat greater than in the 10^{-3} and 10^{-1} groups The difference between the median values (y) is only just significant, even when the 10^{-6} and 10^{-5} groups are combined with the 10^{-3} and 10^{-1} groups[1] It will be noted that the animals in the 10^{-7} group had a slightly prolonged survival time

Challenge dose 10^{-4} (30×10^{3} tubercle bacilli) In the groups challenged with 10^{0} and 10^{-2} there were only few premature deaths (1 and 3 respectively) With the 10^{-4} dose 16 cases were observed (7 with Tub 0 or I and 9 with Tub III) The frequency of premature deaths in the six groups shows only random variations, but the deaths seem to occur at a later date in the 10^{-3} and 10^{-1} groups

1 Standard error $\sqrt{\frac{0\ 0085^2}{37} + \frac{0\ 031^2}{36}} = 0\ 0015$

y difference 0 0156 0 0125 = 0 0031

TABLE 2 d

Survival Times and Degree of Tuberculosis in BCG Vaccinated Red Mice Infected with Different Doses of Bovine Tubercle Bacilli Challenge Dose 10^{-6} 30 tubercle bacilli

Non vaccinated controls		Vaccination dose 10-7 mg BCG		10-6 mg BCG		10-5 mg BCG		10-3 mg BCG		10-1 mg BCG		No vaccination no challenge	
48	IV	2	0	102	III	75	0	26	0	5	0	61	0
53	IV	33	I	110	I	82	I	98	0	73	I	109	0
53	IV	55	IV	125	V	141	V	139	I	110	III	113	0
59	IV	67 decomp		155	V	147	0	174	V	158	I	124	0
64	IV	72	IV	162	I	201	V	181	V	159	0	138	0
65	IV	73	IV	180	III	233	V	218	V	167	IV	176	0
78	V	81	IV	202	IV	235	IV	247	IV	194	I	235	0
88	V	83	V	224	0	260	III	280	IV	197	IV	327	0
90	V	92	IV	243	V	319	V	310	V	238	V	515	0
90	IV	98	IV	243	IV	339	V	408	V	306	V	538	0
95	V	101	IV	245	V	354	IV	411	V	313	V	611	0
96	V	137	0	249	IV	373	V	433	II	332	V	630	0
100	V	147	III	317	V	388	I	465	V	343	V	630	0
105	V	149	I	468	V	467	IV	591	V	365	V	656	0
106	V	176	V	537	V	578	III	719	0	376	IV	719	0
134	V	200	III	581	V	*		*		396	V	738	0
*		225	IV	*		*		*		432	V	761	0
		250	V	*		*		*		527	V	775	0
*		324	V	*		*		*		543	V	***	
**		657	I	*		**		escaped		728	V	***	

The table shows the survival times of the animals in days after challenge
The Roman figures indicate the degree of tuberculosis at time of death
* Died during vaccination challenge interval
* Challenge inoculation unsuccessful
** Died before time of challenge

The survival times for both controls and vaccinated animals were longer than in the 10^{-4} group. The median survival times for the animals vaccinated with 10^{-6} to 10^{-1} mg of BCG varied from 149 to 196 days as against 96 in the control group. The survival times became longer the bigger the dose but there is no significant difference between 10^{-6}, 10^{-5} and 10^{-3} 10^{-1} either as regards median survival time or standard deviation.

Challenge dose 10^{-6} (30 tubercle bacilli). The median survival time for the control animals was the same as with challenge dose 10^{-4}, whereas it was 2 2½ times longer for the vaccinated mice.

For the animals vaccinated with 10^{-6} to 10^{-1} *mg of BCG* the median survival times were 244 to 323, being longer the higher the dose. No significant difference can be demonstrated *between the* individual groups but considered together with the results for challenge dose 10^{-2} and 10^{-4}, it will be seen that there is a *general trend* towards longer median survival times and lower standard deviations.

Closer examination of the survival time distribution in the groups vaccinated with 10^{-7} mg shows that this can be divided into two parts,

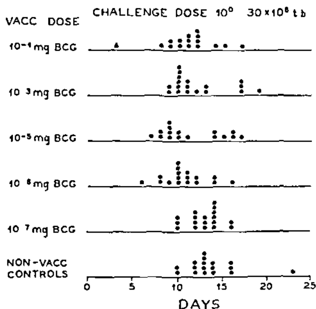


Fig 2 a

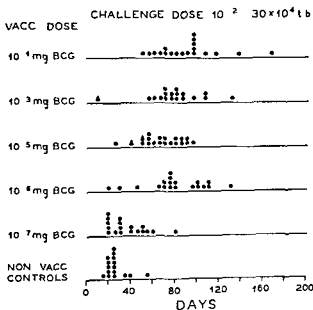


Fig 2 b

viz one with short survival times with distribution as in the control group, and the other with long survival times and distribution as in the groups vaccinated with the higher doses The standard deviation for the control group is $s_y = 0\ 0040$ The median survival time for the 10^{-7} group is significantly higher than the control group, but as the distribution has two modes, it can be assumed that for some of the animals the survival time was significantly prolonged, while for other animals it was unchanged There were many cases of premature death in the vaccinated groups, *viz* 30 altogether (10 with Tub 0, 11 with Tub I, 1 with

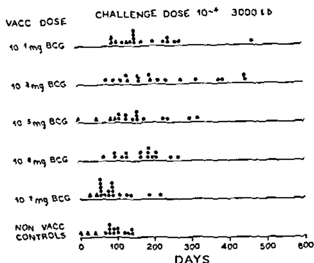


Fig 2 c

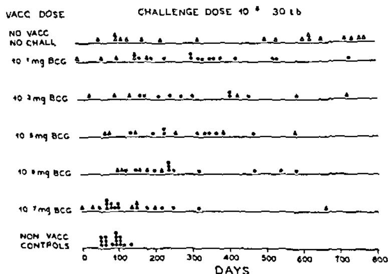


Fig 2 d

Fig 2 a b c and d

Distributi n of survival times of BCG vaccinated red mice after challenge with different doses of bovine tubercle bacilli

△ Animals with tul 0 ▲ Animals with tub I III ● Animals with tub IV V

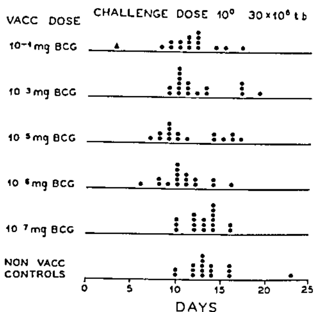


Fig 2 a

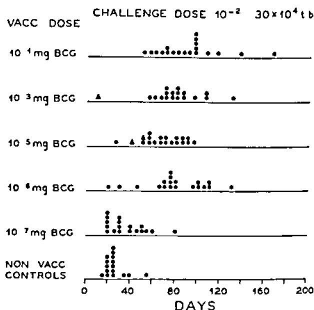


Fig 2 b

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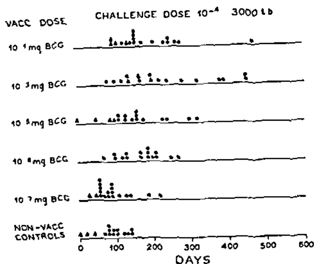


Fig 2 c

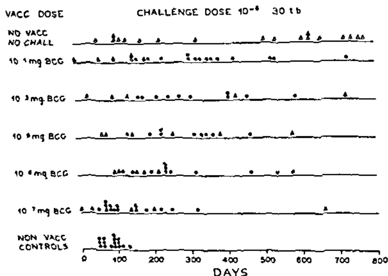


Fig 2 d

Fig 2 a, b c and d

Distribution of survival times of BCG vaccinated red mice after challenge with different doses of bovine tubercle bacilli

△ Animals with tub 0 ▲ Animals with tub I III ● Animals with tub IV V

TABLE 3

Results of Statistical Analysis of Survival Times

Challenge dose	Vaccination dose	Number of animals n	Reciprocal survival times: Arithmetic mean y	Reciprocal survival times: Standard deviation s_y	Median survival time (in days) $\left(\frac{1}{y}\right)$
10^{0}: 30×10^{6} tb	Non-vacc controls	17	0.0753	0.0136	13.3
	10^{-7} mg BCG	18	0.0787	0.0116	12.7
	10^{-6} mg BCG	17	0.0984	0.0245	10.2***
	10^{-5} mg BCG	16	0.0951	0.0265	10.5**
	10^{-3} mg BCG	18	0.0867	0.0188	11.5*
	10^{-1} mg BCG	15	0.0900	0.0176	11.1**
10^{-2}: 30×10^{4} tb	Non-vacc controls	16	0.0406	0.0106	24.6
	10^{-7} mg BCG	18	0.0324	0.0132	30.9
	10^{-6} mg BCG	19	0.0154	0.0098	64.9***
	10^{-5} mg BCG	18	0.0159	0.0071	62.9***
	10^{-3} mg BCG	17	0.0128	0.0026	78.1***
	10^{-1} mg BCG	19	0.0122	0.0036	82.0***
10^{-4}: 3000 tb	Non-vacc controls	12	0.0104	0.0024	96
	10^{-7} mg BCG	17	0.0118	0.0044	85
	10^{-6} mg BCG	16	0.0067	0.0028	149**
	10^{-5} mg BCG	13	0.0061	0.0020	164**
	10^{-3} mg BCG	14	0.0053	0.0032	189***
	10^{-1} mg BCG	14	0.0051	0.0016	196***
10^{-6}: 30 tb	Non-vacc controls	16	0.0131	0.0040	76
	10^{-7} mg BCG	12	0.0099	0.0047	101*
	10^{-6} mg BCG	11	0.0041	0.0019	244***
	10^{-5} mg BCG	9	0.0038	0.0016	263***
	10^{-3} mg BCG	10	0.0036	0.0014	278***
	10^{-1} mg BCG	14	0.0031	0.0012	323***

*, **, and *** denote that the deviation from the control group exceeds the 5 per cent, 1 per cent and 0.1 per cent significance levels respectively

TABLE 4

Relationship between Deviation and Average of the Reciprocal Survival Times

Vaccination dose	Challenge dose: 30×10^{6} tb	30×10^{4} tb	3000 tb	30 tb	Average (excl. 30×10^{6})
Non-vacc controls	0.18	0.26	0.23	0.31	0.27
10^{-7} mg BCG	0.15	0.41	0.37	0.47	0.42
10^{-6} mg BCG	0.25	0.64	0.42	0.46	0.51
10^{-5} mg BCG	0.28	0.45	0.33	0.42	0.40
10^{-3} mg BCG	0.22	0.20	0.60	0.39	0.40
10^{-1} mg BCG	0.20	0.30	0.31	0.39	0.33

Tub II, 7 with Tub III, 1 decayed). Many of the deaths occurred at a late stage (between 100–200 days after challenge) in the same interval in which some deaths were observed among the non-infected control animals.

It will be seen that the standard deviation in the reciprocal survival times (s_y) is lower the lower the average (y) The relative standard deviations (s_y/y) vary less from group to group (Table 4) Except for the highest challenge dose the relative standard deviation seems to be independent of the challenge dose On the whole the groups vaccinated with 10^{-6} mg show the highest and the control groups the lowest values

DISCUSSION

In order to ensure success in vaccination experiments on animals certain conditions must be fulfilled concerning dose of vaccine vaccination challenge interval and challenge dose The requirements as regards vaccine and challenge doses will vary for the individual animal species according to susceptibility of the animals to tubercle bacilli and their ability to develop acquired resistance The question of dosage will be discussed here in relation to white mice guinea pigs and red mice

The technique most generally employed in vaccination experiments with white mice is the same as in the present red mice study i e evaluation of the acquired resistance on the basis of survival time after challenge Since white mice are very resistant to both human and bovine tubercle bacilli it is necessary to use a large challenge dose administered intravenously This must be sufficient to ensure that the control animals die of an acute disease but then it may be so large that the survival time for the vaccinated animals coincides with that of the control animals The dosage range is small and it is difficult to obtain an adequate dose since at time of challenge it is not known with certainty how many tubercle bacilli the dose contains *Swedberg* (15) who has performed many experiments of this kind states that the infection dose must be of such a size that one quarter of the dose injected into

the whole dose

With another technique *Dubos & Pierce* (3) have been able to use a small challenge dose They challenged vaccinated white mice intravenously with 0.2×10^{-5} ml culture of a bovine strain sacrificed the animals after a certain period and determined the number of virulent tubercle bacilli in the spleen by quantitative culture They found a clear difference between a weakly virulent and a more virulent BCG strain in their ability to restrict multiplication of bacilli

Schwabacher & Wilson (11) stress the importance of a suitable relationship between dose of vaccine and test dose They vaccinated particoloured mice with living BCG or heat killed human tubercle bacilli in doses from 800 000 to 2 *milliard* and challenged the animals with 1 100 million virulent human tubercle bacilli Statistical analysis of the material which comprised 667 mice showed that the survival time

was significantly[1] larger in the vaccinated than in the non-vaccinated animals when the vaccine dose was large (*20 million bacteria or more*) and the infection dose also large (100 million bacteria) There was no significant difference in the survival time in any experiments in which the vaccine dose was less than 20 million bacteria or in which the infecting dose was relatively small (1–10 million bacteria)

The effect of vaccination with heat-killed vaccine is apparently greater in white mice (and closely-related mice) than in guinea pigs *and red mice In the above-mentioned study of Schwabacher & Wilson* no difference could be found between the effect of living BCG and heat killed human tubercle bacilli Correspondingly, *Swedberg* found that heat-killed human tubercle bacilli were just as effective as living BCG but that heat-killed BCG gave poorer protection *Battignani & Ciaccia* (1) report that tubercle bacilli killed by formol had a stronger effect than living BCG and heat-killed tubercle bacilli, the latter two being almost equally effective

The presence of a large number of living BCG in the organism of white mice is in itself sufficient to give protection against a high infection dose *Dubos & Pierce* (3) found a definite prolongation of the survival time in mice vaccinated intravenously with large doses and infected two days later with 0 2 ml of a bovine culture Under these conditions there can be no question of acquired resistance in its general sense

While the effect of vaccination with a killed vaccine against a high challenge dose may be as great as with a living vaccine in white mice, living BCG gives a greater acquired resistance than killed vaccine in red mice (4, 7) (The challenge dose in these studies was small)

It is the opinion of the writers that the same applies to guinea pigs (for references, see *Jespersen & Magnusson* (7)) In recently published experiments *Weiss* (16) and *Weiss & Wells* (17, 18) examined on guinea pigs the effect of phenol-killed tubercle bacilli, as well as a methanol extract and the extraction residue The animals were challenged *with various doses intraperitoneally, intramuscularly* or by inhalation, and the survival times were recorded *Weiss'* interpretation of the results is that non living vaccines can consistently induce as high a degree of acquired resistance as living BCG

It is not, of course, permissible from experiments on white mice infected with giant doses (about 1–10 million tubercle bacilli injected into an animal weighing 20 g) to draw conclusions concerning the value of a vaccine for use in humans Nor can the value of killed versus living vaccines be elucidated by such studies

In vaccination experiments on guinea pigs the size of the challenge dose is of less significance for the demonstration of a vaccination effect

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[1] The significance level is 0 3 per cent

However, here also a too strong test infection may obscure the acquired resistance.

Swedberg, using the intravenous challenge route, demanded a similar criterion for the test dose for guinea pigs as for white mice (as mentioned above). The dose generally used in his experiments was 0.004 mg culture of the bovine strain Ravenel. That dose, which is near to the permitted limit, caused death of the control animals after about 30 days and of the BCG vaccinated after about 40 days. With a challenge dose of 10^{-6} mg of bovine tubercle bacilli the median survival time for non-vaccinated guinea pigs was 82 days and for BCG vaccinated 109–124 days (*Jespersen* (6)).

Bjerkedal (2) examined the effect of 0.1 ml of standard strength Danish BCG on guinea pigs challenged intraperitoneally with different doses of H37 Rv. The effect, expressed as the ratio of the median survival time of the vaccinated animals to that of the controls, was the same for doses containing from millions down to a few hundred bacterial units. Infection with 5.9×10^{2}, 5.9×10^{4} and 5.9×10^{6} units resulted in median survival times of 25, 60 and 110 days respectively for the non vaccinated, and 65, 145 and 240 days for the vaccinated animals.

In red mice, just as in guinea pigs, there is a large challenge dosage range within which the effect of BCG vaccination manifests itself in the form of prolongation of the survival time.

Against challenge doses of from 300 000 to 30 tubercle bacilli, doses of BCG vaccine down to 10^{-6} mg induce a definite increase in the resistance of the animals. The median survival times after a given challenge dose are longer the larger the vaccine dose, but there is no question of any great difference in the degree of acquired resistance in animals vaccinated with 10^{-6} and 10^{-1} mg of BCG. Similar results have been obtained in experiments on guinea pigs (6, 11).

A challenge dose of 300,000 bacteria killed the non-vaccinated red mice after 25 days and the vaccinated after 65–82 days. The difference in median survival times was great and the upper limit for the permissible challenge dose was not yet reached. When a 100 times lower dose was used a corresponding degree of effect was achieved. The median survival times for controls and vaccinated animals were 96 and 149–196 days.

The greatest difference between the survival times of vaccinated and non vaccinated animals was found with the lowest challenge dose (30 bacteria). The control animals died at the same time as those which had been given a dose 100 times larger, whereas the survival times for the vaccinated in the two groups were 149–196 and 244–323 days respectively. (In two previous experiments (13, 9) it was found that the survival time of red mice infected intravenously with virulent bovine tubercle bacilli was independent of the dosage with doses less than 100–200 bacteria.)

Evaluation of the median survival times of vaccinated animals in-

fected with the lowest challenge doses is made difficult by the occurrence of the premature deaths The majority of these took place between 100 and 200 days after challenge, *i e* in the same period during which there was an accumulation of deaths in the non-infected control group They occurred with the lowest challenge dose in all the vaccinated groups but at the next challenge dose only in the groups vaccinated with 10^{-3} and 10^{-1} mg of BCG Since the calculation of median survival times includes only the animals with well-developed tuberculosis, this means that for the lower challenge doses the effect of the vaccine is under estimated

It cannot be determined with certainty whether in particularly favourable circumstances BCG vaccine may induce a resistance sufficient to prevent the development of progressive tuberculosis The two animals which lived longest in the groups vaccinated with 10^{-7} mg and 10^{-1} mg of BCG, both challenged with the lowest dose, died after 657 and 719 days

Autopsy of the first mouse revealed no specific changes Culture from liver and lungs was negative, but there was growth of a few colonies in the spleen The other mouse, which died on the 719th day, showed no signs of tuberculosis either on autopsy or by culture Despite these findings, it is reasonable to assume that some of the tubercle bacilli injected survive somewhere or other in the body of the animal and will start to multiply when a favourable opportunity occurs

With the lowest challenge dose, it was possible to demonstrate a significant prolongation of the survival time after vaccination with 10 mg of BCG, which was not the case with the higher challenge doses The animals vaccinated with 10^{-7} mg can be divided into two groups, one where death occurred from 55–101 days after challenge as in the control group, and another where the survival times varied between 137 and 657 days, with a distribution as in the groups vaccinated with the larger doses On the basis of the colony counts, the inoculum of vaccine per animal contained 1–2 units The figure represents a minimum value Culture was carried out on Lowenstein-Jensen medium, and it is known that, for technical reasons, culture on that medium gives lower counts than on Dubos' oleic-acid-agar plates Despite this, there may have been some cases where the inoculum did not contain any BCG bacteria whatsoever The reason why only about half the animals showed prolonged survival times may be that the vaccination period was not long enough for the acquired resistance to have developed in some animals, or that the threshold value varies slightly from animal to animal

The smallest doses of BCG did not generally seem to be able to induce "maximum" resistance The falling tendency in the survival times of mice vaccinated with doses from 10^{-1} to 10 [illegible] mg of BCG presumably continues in the 10^{-7} group with the prolonged survival times However, it is difficult to evaluate the median survival time in the latter group as 4 of the 8 animals died prematurely Even though the effect of vac-

cination with 10 mg of BCG did not reflect itself in the median survival times with challenge doses 10^{-4} and 10^{-7}, it did find expression in another way, in that the relative standard deviation was greater in the vaccinated than in the non-vaccinated groups

Despite the fact that a weak acquired resistance was most clearly demonstrated with a very small challenge dose, doses of up to at least 800,000 bacteria can be used in experiments where various BCG strains are to be compared (*Jespersen & Bentzon,* to be published)

In the present study, a challenge dose was not obtained which resulted in equally long survival times for the vaccinated and non-vaccinated animals After challenge with the undiluted suspension, the vaccinated animals had a shorter survival time than the controls A shortening of the survival time could be demonstrated in all groups vaccinated with doses down to 10^{-6} mg, *i e* in the same groups where a marked resistance was found with the smaller challenge doses

It is a common trait in both red mice and guinea pigs that vaccination with BCG causes an acquired resistance which can be demonstrated in the form of prolonged survival time after intravenous challenge doses varying from one tubercle bacillus up to several hundred thousand However, the acquired resistance is stronger in red mice than in guinea pigs As mentioned, the median survival time with a challenge dose of 10^{-6} mg of bovine tubercle bacilli was 82 days in non vaccinated and 109–124 days in BCG vaccinated guinea pigs In the present study, the median survival time after infection with the 10^{-6} dilution was about the same for the non-vaccinated mice (76 days), while for the vaccinated *mice it* was more than twice as long as for the corresponding guinea pigs (244–323 days) There is a difference between red mice and *guinea pigs* in another respect also The smallest dose of BCG vaccine required *to induce* acquired resistance in almost all the animals in a group is lower in red mice than in guinea pigs After subcutaneous vaccination *and a three-month* vaccination period, the threshold value lies at about 200 *bacterial units* for guinea pigs (8, 10), and at 10 15 units for red mice

SUMMARY

The aim of the experiment was to determine the range of challenge dosage *which permits* demonstration of acquired resistance in BCG vaccinated *red mice* Four groups of 100 red mice were vaccinated with 10^{-} 10^{-4}, 10^{-3}, 10^{-2} or 10^{-1} *mg of living* BCG, each dose being administered to 20 animals per group After three months the four groups and non-vaccinated *control groups* were challenged intravenously with 0 2 ml of dilutions 10^{1}, 10^{-2}, 10^{-4} and 10^{-6} *of an* ultrasonic-treated Dubos culture of bovine tubercle *bacilli* The mice were observed until they died spontaneously The survival *time in days after* challenge was used as indication of the degree of acquired *resistance*

Against challenge doses of from 30 to 300,000 tubercle bacilli, vaccinc

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The aim of the experiment was to determine the range of challenge dosage which permits demonstration of acquired resistance in BCG vaccinated red mice Four groups of 100 red mice were vaccinated with 10^{-7} 10^{-6} 10 [illegible] 10^{-3} or 10^{-1} mg of living BCG, each dose being administered to 20 animals per group After three months the four groups and non [illegible] intravenously with 0 2 ml of [illegible]asonic treated Dubos culture [illegible]bserved until they died spon[illegible] challenge was used as indi[illegible]cation of the degree of acquired resistance

Against challenge doses of from 30 to 300 000 tubercle bacilli, vaccine

doses from 10^{-6} mg (12 units) to 10^{-1} mg of BCG (1.2×10^{6} units) caused a definite prolongation of the survival time. The effect of vaccination could be demonstrated with the same degree of certainty over the whole range.

The effect of vaccination with 10 [illegible] mg of BCG, *i.e.* only few BCG units was demonstrable only with the lowest challenge dose.

With a fixed challenge dose, the median survival times increased the larger the vaccine dose, but the degree of acquired resistance was not particularly larger in animals vaccinated with 10^{-1} mg of BCG than in those vaccinated with 10^{-6} mg of BCG.

As regards the highest challenge dose (30 million tubercle bacilli), a paradoxical vaccination effect was observed, the survival time of the vaccinated animals being shorter than that of the non-vaccinated. The reduction could only be observed in those groups of animals where a definite resistance could be found with the lower challenge doses, and must thus have some connection with the allergic condition of the animals.

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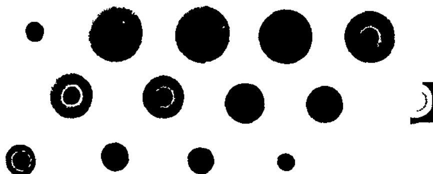


Fig 1

Haemolyzed zones on blood agar plate corresponding to 2 fold serial dilutions of haemolysin produced by *Bacillus cereus* Diluent saline The plate is photographed in transmitted light after incubation at 37° C for 13 hrs

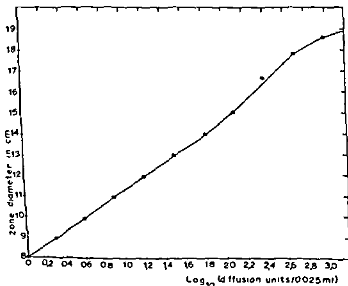


Fig 2

Logarithmic standard curve for haemolysin from *Bacillus cereus* (ref Table 1 and Fig 1) The diameters are measured after 10 × magnification Diluent saline

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SEPARATION OF HEMOLYSIN AND EGG YOLK TURBIDITY FACTOR IN CELL-FREE EXTRACTS OF BACILLUS CEREUS

By

KÅRI FOSSUM

Received 30 iii 63

McGaughey & Chu (1948) studied the egg yolk curd-forming activity and the haemolytic activity in culture filtrates of certain strains of *Bacillus cereus*. These authors observed that in most of the strains these two activities were closely parallel. A few strains, however, exhibited a relatively much higher ratio of haemolytic activity. They concluded that "Although there is no direct evidence to show that haemolysis is due to the phospholipinase activity, indirect evidence strongly suggests that part, if not all, of the haemolysin of *Bacillus cereus* and *Bacillus mycoides* is closely correlated with the yolkreacting substance or phospholipinase".

Ottolenghi et al. (1961) found that phospholipolytic and haemolytic activities showed different sensitivity to heat. After incubation at pH 8.2 at 56° C for 20 minutes, more than 95 per cent of the haemolytic but less than 50 per cent of the phospholipolytic activity was lost. They found, further, that under certain conditions of growth only haemolytic activity was manifested.

The latter work thus indicates that the two activities are probably associated with two different enzymes.

The present work was undertaken for the purpose of demonstrating whether the enzyme or enzymes responsible for the two activities could be separated by means of column chromatography. For comparison, attempt was also made to differentiate between these and certain other enzymes.

MATERIALS AND METHODS

Organisms. By preliminary studies those strains of *Bacillus cereus* which yielded the best enzymatic activities were selected. The strains were isolated and classified by the Department of Food Hygiene and Microbiology, Veterinary College of Norway, and were kept in freeze dried state. In this paper only two strains are considered:

Bacillus cereus NVH 322
Bacillus cereus NVH 1222

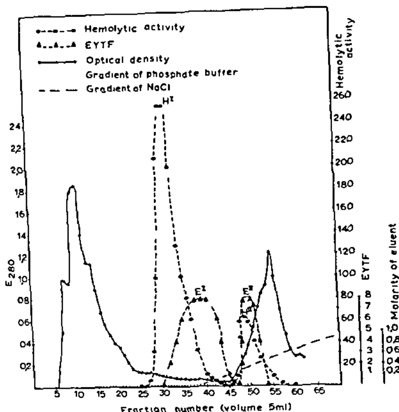


Fig 4

Combined phosphate buffer and NaCl gradient elution of hemolytic activities (HI and HII) and EYTF s (EI and EII) from DEAE-cellulose columns at 4 5° C The enzymes originated from *Bacillus cereus* (NVH 322)

tion against large quantities of tap water and subsequently distilled water Merthio-

used

Agar (Difco Bacto Agar 0140–01) 1 4 per cent
NaCl sufficient to get a final concentration of 0 95 per cent
Washed bovine erythrocytes 4 per cent
Merthiolate to a final concentration of 1 10000
pH 6 5

The red corpuscles were added to the base medium at a temperature of 46° C After mixing the medium was poured on glass plates with plastic frame to form a layer of 2 mm During pouring the plates were placed exactly horizontally

In these agar plates circular wells of 6 mm diameter were cut with a cork borer at sufficient intervals to avoid interference between the haemolyzed zones Into these wells 0 025 ml of the enzyme preparation to be investigated was transferred with a micropipette according to *Sandvik* (1959) The blood agar plates were then incubated at 37° C for 13 hrs At the end of the incubation period the plates were

TABLE 1

Diameters of Haemolyzed Zones for Double Dilutions of Haemolysin Produced by Bacillus cereus and the Corresponding Units

Dilutions	Zone diameters in cm (10 times magnification)	Units of haemolysin
1	19 0	2048
1 2	18 5	1024
1 4	17 8	512
1 8	16 7	256
1 16	15 0	128
1 32	14 0	64
1 64	13 0	32
1 128	12 0	16
1 256	11 0	8
1 512	10 0	4
1 1024	9 0	2
1 2048	8 0	1

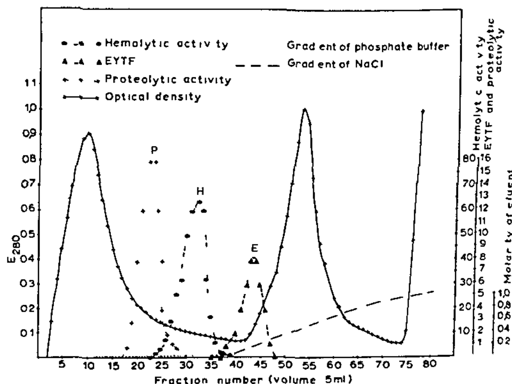


Fig 3

Combined phosphate buffer and NaCl gradient elution of proteolytic activity (P) haemolytic activity (H) and EYTF (E) from DEAE cellulose columns at 4 5° C The enzymes originated from *Bacillus cereus* (NVH 1222)

After incubation the liquid medium was centrifuged at approximately 25000 × g for 15 20 minutes after which the enzyme preparation was concentrated by salting the supernatant by addition of ammonium sulphate to 80 per cent saturation The preparation was allowed to stand in the refrigerator over night after which the precipitate was collected by high speed centrifugation The precipitate was then suspended in a small volume of distilled water and dialyzed under constant agita

DISCUSSION

As shown in Fig 3, the haemolytic enzyme and the EYTF could be distinctly differentiated

This indicates that the two activities are in this case due to two different enzymes

In Fig 4, one peak of haemolytic activity (H[I]) and one peak of EYTF (E[I]) were found to correspond with the peaks H and E in Fig 3 In addition one more peak of each of these two activities was demonstrated (H[II] and E[II]) It was not possible to differentiate between these latter peaks of haemolytic activity and EYTF (H[II] and E[II]) by the method used The possibility cannot therefore be excluded that the two activities in this case may be due to the same enzyme But no attempt has been made to verify this

It should be noted that, when the experiments were repeated with the same strain under apparently identical conditions, the latter peaks only occurred occasionally The two first peaks, on the other hand, were always reproducible for all strains examined

When, as in this case, there occur two different components of the activities in question, this may be due to two quite different enzymes or to the occurrence of active fragments derived from a major enzyme molecule by means of proteolytic enzymes With regard to haemolytic activities and EYTF in the present case, the problem is as yet not clear, but it seems as if the latter theory is the more probable The occurrence of active fragments may thus be due to the action of the proteolytic enzymes present in the enzyme mixture

The enzymes derived from the first experiment (Fig 3) were used for further investigations, (*Fossum*, unpublished) because of the highly clearcut peaks obtained in this case

It is generally assumed that in culture filtrates of *Clostridium perfringens*, the lethal, the dermonecrotic, the haemolytic and the egg yolk turbidity factors are due to a single toxin This toxin can therefore be assayed by means of the turbidity produced in egg yolk solution (*van Heyningen* 1951)

The researches of *Kushner* (1956) lend additional support to the belief that, in *Clostridium perfringens* preparations, turbidity production in egg yolk, phospholipase action on egg yolk and lecithinase activity are all caused by the same enzyme On the other hand, *in the case* of *Bacillus cereus* they found that turbidity production and lecithinase activity had distinctly different heat stabilities *Autoclaving completely* destroyed both lecithinase and phospholipase activity, but turbidity in egg yolk saline was still produced *This indicates* that lecithinase activity and egg yolk turbidity factor are dependent on two different enzymatic activities

In the present inquiry only the egg yolk turbidity factor has been

photographed in perpendicularly transmitted light The negatives were projected on a screen and the zone diameters measured at 10 times magnification as described by *Sandvik* (1962)

When enzymes diffused into the medium clear zones of haemolysis occurred *around the wells* The boundary between the haemolyzed zone and the surrounding medium was very sharp By transferring twofold dilutions of enzyme into series of *wells* a standard curve could be obtained by measuring the corresponding zone diameters on the plates For practical reasons the highest dilutions of the haemolysin *that* gave a clear zone in the blood agar was defined as *one diffusion unit* A standard curve was drawn by plotting the zone diameters against the logarithms of the number of diffusion units in 0 025 ml diluent (Fig 1 and 2 and Table 1)

This type of standard curve was under certain standard conditions used for determining the concentration of enzyme in a sample By comparing the zone diameter and the sample in question with the corresponding standard curve the enzyme concentration could be determined The following conditions must be observed for this kind of determination The standard dilutions and the unknown sample must be applied on the same blood agar plates, or on identical plates made at the same time A standard curve made for quantitative determinations must be prepared on the basis of the same enzyme extract and, as far as possible under the same conditions as the enzyme to be determined

For demonstration of EYTF, equal parts of 3 per cent nutrient agar and a 10 per *cent egg yolk suspension in nutrient broth* were used *The egg yolk suspension* had previously been centrifuged at 900 g for 10 minutes in order to remove large particles The agar and the egg yolk suspension were mixed at 46° C and poured on plates as described for the blood agar plates These plates were incubated for 48 hrs prior to reading

The proteinase was demonstrated and titrated as described by *Sandvik* (1962)

Column chromatography Columns were packed as described by *Peterson & Sober* (1956) A commercial product of diethylaminoethylcellulose (DEAE cellulose) produced by Eastman Kodak was used The packed columns were 1 2 cm in diameter and length 20 cm The column was placed in a refrigerator at +4° C Arrangements for gradient elution were made as described by *Hurlbert* et al (1954) In order to get a constant flow rate a micropump was coupled to the system The flow rate was 15 20 ml/hr As eluent phosphate buffer of pH 6 5 was used After some time the buffer gradient was supplemented with NaCl (Fig 3 and 4) The effluents were collected in fractions of 5 ml

Before use the columns were equilibrated with distilled water The amount of protein applied was as a rule 150–200 mg

OBSERVATIONS

In Figs 3 and 4 are shown the results of two experiments As will be seen, proteolytic activity was only determined in one of the said experiments (P in Fig 3)

It will be noticed that in both cases the majority of contaminating substances were clearly separated from the enzymes in question

Fig 3 shows the results obtained with cell free extract of one strain of *Bacillus cereus* (NVH 1222) after 2 days incubation In this case one distinct and differentiated peak of each of the two enzymes was obtained (H and E)

Fig 4 shows the results obtained with *Bacillus cereus* (NVH 322) after 24 hrs incubation Also in this experiment the peaks corresponding to the haemolytic activity (H) and the EYTF (E) shown in Fig 3 were obtained (H^I and E^I respectively) In addition, however, additional peaks of both haemolytic activity and EYTF, which were fully confluent (H^{II} and E^{II}), were obtained in this case

*THE BLOOD BANK AND BLOOD GROUPING LABORATORY
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PROBLEMS IN BLOOD GROUPING ASSOCIATED WITH AN ABNORMALLY HIGH CONCENTRATION OF SPECIFIC BLOOD GROUP SUBSTANCE IN THE SERUM

By

HANS HOSTRUP

Received 26. ix. 63.

It is of importance to identify sources of error in blood grouping and compatibility tests in order, if possible, to eliminate these in the routine techniques used in the laboratory.

Within the last four years it has appeared that simple tests for blood grouping and compatibility may fail if the serum contains large amounts of specific A or B blood group substance. Suspensions of unwashed blood cells prepared from such samples may contain so large quantities of substance that even strong test sera and recipient sera may be neutralized and therefore fail to agglutinate the blood cells.

Group specific A and B substance occurs in the serum of individuals of the groups A, B and AB. Normally the concentration is so low that the substance can be demonstrated by the inhibition technique only when highly diluted anti-sera are used (*Hostrup* 1962).

The specific substance inhibits the blood cell agglutination produced by homologous antibodies. The inhibition which normal A, B and AB sera exert on homologous antibodies is so weak that it is without practical importance in serological blood grouping work. In cases in which the substance occurs in large amounts, special precautions must be taken.

We have had an opportunity to study five cases in which large quantities of blood group substance in the serum caused difficulties in blood grouping. We find that it is justified to publish these cases partly in order to discuss why blood group substance occasionally occurs in high concentrations in the serum, and partly in order to emphasize the measures which should be taken to avoid serological complications in blood grouping.

Aided by grants from *Købmand i Odense Johann og Hanne Weimann f. Seedorffs Legat* and *Gårdejer P. L. Pedersens Legat*.

examined No attempt has hitherto been made to ascertain whether this activity is identical with the phospholipase activity

SUMMARY

1 An agar plate method for qualitative and quantitative determination of haemolytic activity and egg yolk turbidity factor (EYTF) has been described
2 Column chromatography was employed in order to separate haemolytic activity and EYTF in cell free extract of *Bacillus cereus*
3 In most cases the haemolytic activity and EYTF could be distinctly separated by the chromatographic procedure Occasionally however, an additional fraction occurred which possessed both the activities
4 The possibility of active fragments of the two enzymes cannot be excluded

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PRESENT CASES

Case 1

The patient H P was a man aged 63 group B, Rh D negative Le (a−b+), secretor who was admitted to hospital with intense jaundice on Nov 30 1960 [illegible] the size of a clenched fist, [illegible] d

[illegible]

ened further and death occurred on Jan 8 1961 Autopsy was not performed

Blood group determination on an Eldon card (method II) performed on Dec 7 in a local hospital gave a questionable result with doubtful agglutination in the anti B field for which reason a sample was submitted to our Blood Grouping Laboratory for further investigation Blood grouping with the ordinary technique [illegible] weak agglutination with anti B and none [illegible] with anti B The [illegible] (method II) con[illegible] d with unwashed blood cells whereas washed blood cells gave strong agglutination

The concentration of B substance in the serum was studied in seven different samples by means of the inhibition technique using strong anti B diluted to 1 4 (Table 1) The concentration of B substance was also studied in bile and saliva

TABLE 1

Concentrations of Blood Group B Substance in the Serum Saliva and Bile from Case 1 and in the Serum from a Normal B Donor Determined by Inhibition Titrations

Samples investigated	Serum Dec 7	Serum Dec 12	Serum Dec 14	Serum Dec 19	Serum Dec 23	Serum Dec 28	Serum Jan 5	Saliva	Bile	Serum Normal control
Highest dilution capable of inhibiting anti B completely	256	256	1024	2048	512	512	512	64 000	36 000	0

The serum concentration of B substance was so high that it in the dilution 1 256 neutralized equal parts of the antibody used Minor fluctuations in the substance concentration were observed from sample to sample B substance was present in the bile and saliva in still higher concentrations

Case 2

A man S M aged [illegible]
n Sept 27 [illegible]
ously subt[illegible]
The liver w[illegible]
Jaundice wa[illegible] and there were no signs of ascites The acute symptoms subsided during the observation period

As the result of the blood group determination on an Eldon card (method II) performed in the local hospital was questionable with only weak agglutination in the anti B field a sample withdrawn on Sept 29 [illegible] grouping [illegible]
technique [illegible]

[illegible]

[illegible] Oct 5 [illegible]
serum (4 [illegible]
neutralize[illegible] parts of the antibody Serum from the other two samples in the dilutions 1 1 and 1 2 neutralized the antibody B substance was not present in excessive amounts but a transient moderate increase was observed

Previously Reported Cases

The first report on abnormally high concentrations of blood group substance in the serum was published by *Barber & Dunsford* (1959) The blood group could not be determined on a suspension of unwashed blood cells from a patient with metastasizing carcinoma of the stomach, because the serum contained large amounts of A substance The patient was a secretor. The authors discussed if the large amounts of substance was referable to the gastric cancer, if it might be the cause of the disease; and if the high serum concentration of the substance was compatible with the survival of the patient The authors studied sera from 500 donors of group A by means of a fairly coarse technique and found one case in which substance occurred in a higher concentration than among the others

Moullec et al (1960) found large amounts of substance in the serum of a patient with a malignant mucinous cystoma of the ovary One litre of ascitic fluid was present Substance was revealed in high concentrations in the ascitic fluid, tumour tissue, cyst fluid and saliva The authors expressed the view that the blood-group substance was produced by the tumour, secreted into the peritoneal cavity and then resorbed into the blood stream

Almost simultaneously, *Salmon & Malassenet* (1960) reported two similar cases observed in a group-A patient suffering from cancer of the pancreas with metastases to the liver and a clinically healthy group-B donor, both were secretors

Freiesleben et al (1961) described two cases in which blood grouping had caused difficulties Both patients were of group A and had a mucinous cystoma of the ovary In the first case, the first examination revealed large amounts of A substance in the serum, while repeated examination 18 days later when the cyst was removed by operation showed an almost normal concentration On the first postoperative day the concentration was again somewhat increased Ascites was absent The second patient revealed large amounts of A substance in the serum on the day of operation Considerable ascites was present During the weeks after operation the concentration in the serum decreased Both patients were secretors

Finally, *Hatton & Walsh* (1961) reported an additional case with increased amounts of substance in the serum in a patient with a mucinous cystoma of the ovary Three days before operation, the patient's unwashed blood cells showed only weak agglutination with anti-B and on the first day after operation no agglutination at all Six days later, greatly increased amounts of B substance were demonstrated in the serum After another month, the concentration showed an appreciable fall, but was still increased This patient was also a secretor The authors expressed the opinion that the blood group substance passed into the blood stream direct from the cyst or through the peritoneal cavity and the lymphatics

in local hospitals were questionable Here the reactions with our test sera which fulfil the minimum requirements set up by the National Institute of Health (U S A) were weak in Case 1 but strong in Cases 2 and 3 In the determination on Eldon cards the suspension used is stronger (approx 15 per cent) than that employed with the ordinary technique (5 per cent) In the stronger suspension used on Eldon cards the blood group substance in the serum (or plasma) may exert a greater influence Attempts were made to clarify if this was the cause of the greater difficulties with the Eldon cards or if these were due to the use of too weak reagents on the cards

Blood cell suspensions with a varying content of substance were prepared To washed packed A and B blood cells were added equal parts of A or B substance (Knickerbocker Biosales Inc) or substance diluted with A and B serum respectively in the ratios of 2 1 1 2 and 1 3 The suspensions were shaken for 1 hour and then allowed to stand at 4° C for 12 hours for sedimentation From the sediment was prepared first an approx 15 per cent suspension of three drops of blood cells and 12 drops of saline and from this in turn a 5 per cent suspension by dilution with saline

ABO grouping was then performed on all the suspensions both on Eldon cards with the 15 per cent suspension and on glass tiles with both the 15 per cent and the 5 per cent suspension The test sera used were the dried reagent on the Eldon cards and one drop of anti A and anti B on the glass tiles The Eldon cards were allowed to stand for 1 minute and then rocked for 1 minute before reading while the reactions on the glass tiles were read after standing at room temperature for 10 minutes

The agglutination of the 15 per cent suspension decreased markedly with increasing concentration of the blood group substance in the serum both on the Eldon cards and the tiles whereas the influence of the substance concentration on the agglutination of the 5 per cent suspension was less pronounced (Table 3)

The results suggest that the difficulties were not due to weak reagents but might be explained by the greater amount of blood group substance in the 15 per cent suspension than in the 5 per cent suspension

In Case 2 there was some haemolysis of the first blood sample with a content of free haemoglobin of 100 *mg %* in the serum In order to throw light on the influence of haemolysis of blood samples on the con-centration of bl

lysates of A₁

A and B b

of equal amo

... were frozen to —20° C and again allowed to thaw out They were then centrifuged at 3 000 r p m for 20 minutes after which the supernatant was pipetted off A number of

The first blood sample (Sept 29) was slightly haemolysed The concentration of free haemoglobin in the serum was 100 mg% Visible haemolysis was absent in the last two samples

Case 3

A healthy female donor, aged 37 group A_1 Rh D positive Le(a—b+) secretor In the local blood bank blood group determination on Eldon card (method II) performed on a sample stabilized with heparin showed questionable agglutination in the anti A field A sample was therefore submitted to our Blood Grouping Laboratory, where blood grouping with the ordinary technique using unwashed cells showed strong agglutination with anti A test serum The plasma of the sample contained anti B On Eldon cards unwashed blood cells showed weak agglutination in the anti A field whereas washed blood cells yielded normal strong agglutination The suspension of unwashed blood cells was prepared from the blood cells of the sample after centrifugation so that the plasma was largely removed from the blood cells

The concentration of A substance in the plasma was higher than that in the serum from a normal A donor The concentration in the plasma from a new sample obtained four months later was normal In order to study if the addition of heparin could explain the increased concentration of substance in the plasma an experiment was performed in which the ability of a heparin solution to inhibit anti A was tested but the experiment was negative The concentrations of substance were determined by means of the inhibition technique using a strong anti A diluted to 1 5 and 1 4 (Table 2)

TABLE 2

Concentrations of Blood Group A Substance in the Plasma and Saliva from Case 3 in a Heparin Solution and in the Serum from a Normal A Donor

Samples investigated	Plasma Aug 29	Plasma Feb	*Saliva*	*Heparin*	Serum Normal control
Highest dilution capable of *inhibiting anti A completely*	*8*	*0*	*128*	*0*	0

Case 4

A newborn male infant P group B Rh D positive secretor Mother's blood group was A Rh D negative Coombs' direct test performed on the infant's blood cells was negative In the blood group determination performed on cord blood with the ordinary *technique in tubes it was found that the blood cells did not agglutinate* with anti A or anti B whereas washed blood cells showed strong agglutination with anti B The serum from the sample in the dilution 1 128 neutralized equal parts of strong anti B test serum Unfortunately no further blood samples were secured

Case 5

A newborn female infant P group A Rh D negative Secretor Mother's blood *group was O Rh D negative The infant was delivered by Caesarean section* because of prolapse of the umbilical cord Coombs direct test on blood cells from the cord blood was negative With the ordinary technique in tubes a suspension of unwashed blood cells from the cord blood did not agglutinate with anti A or anti B *while washed blood cells showed strong agglutination with anti A*

The content of A substance in the serum was studied by the inhibition technique with diluted anti A (4 agglutinating units) Complete neutralization of the antibody was observed by the serum dilution of 1 256 The serum from a new blood sample taken on the second postnatal day neutralized equal parts of the antibody *in the* dilution 1 2

In Cases 1, 2 and 3, the blood samples were submitted to our Laboratory because the blood-group determinations performed on Eldon cards

strated in the weaker solutions which contained an appreciable amount of blood group antigen (Table 4)

The fluid from mucin containing cysts contains large amounts of specific blood group substance when the patient is a secretor (*Morgan & van Heyningen* 1944) However the presence of large amounts of substance in an organ does not necessarily involve that the concentration in the serum is higher than normal

Sera from three patients were studied *viz* two with mucinous ovarian cysts and a third with a cystic teratoma of the thymus All three were secretors and in all three cases did the cyst fluid contain large quantities of specific blood group substance However the concentration of the blood group substance in all three sera was normal (Table 5) Ascites was absent in the two patients with ovarian cysts and the patient with a thymic cyst did not suffer from pleural effusion

TABLE 5

Concentrations of Blood Group Substance in the Serum and Cyst Fluid from two Patients with Mucinous Ovarian Cysts and one with Teratoma of the Thymus Determined by Inhibition Titrations

Samples investigated			Cyst fluid	Serum
Highest dilution capable of inhibiting anti A or anti B completely	Patient 1 AB Le(a—b+) Secretor	Mucinous cyst of ovary	A 4096 B 16 000	A 0 B 0
	Patient 2 A Le(a—b—) Secretor	Mucinous cyst of ovary	A 256	A 4
	Patient 3 A Le(a—b+) Secretor	Teratoma of thymus	A 32 000	A 4

DISCUSSION

The investigations reported here suggest that the *increased* amounts of blood group substance in the serum may differ in origin

Four of the previously reported cases occurred in patients with mucinous cysts of the ovary Operation revealed ascitic fluid in two but not in the third while no information on this point is available in the fourth case The three patients with mucin containing cysts considered here all revealed large quantities of specific substance in the cyst fluid but normal concentrations in the *serum* In none of these cases was ascites or pleural effusion present It is reasonabl[illegible]

[illegible] substance is carried through the

TABLE 3

A_1, A_2 and B Red Cells Were Suspended in Serum with Various Concentrations of Specific Blood Group Substance. After Sedimentation 15 per cent and 5 per cent Red Cell Suspensions in Saline Were Prepared from these Samples. The Agglutination of a 15 per cent Suspension on Eldon Cards and Glass Tiles Was Compared with that of a 5 per cent Suspension on Glass Tiles

Dilution of blood group substance in the serum	15 per cent suspension of A_1 red cells		5 per cent suspension of A_1 red cells
	Eldon card	Tile	Tile
Undiluted	—	—	++
2 1	—	—	++
1 2	+	+	+++
1 3	+(+)	+(+)	+++(+)
	15 per cent suspension of A_2 red cells		**5 per cent suspension of A_2 red cells**
	Eldon card	Tile	Tile
Undiluted	—	—	—
2 1	—	—	+(+)
1 2	—	—?	++
1 3	—?	(+)	+++
	15 per cent suspension of B red cells		**5 per cent suspension of B red cells**
	Eldon card	Tile	Tile
Undiluted	+	+	++
2 1	+(+)	+(+)	+++
1 2	++	++	+++
1 3	++	++	+++(+)

TABLE 4

Concentrations of Substance Inhibiting anti A and anti B in Various Dilutions of Haemolysed A and B Red Cells Compared with the Concentrations of Free Haemoglobin

Haemolysed A_1 red cells						
Dilution	1 8	1 16	1 32	1 64	1 128	1 256
Highest dilution capable of inhibiting anti A completely	64	32	16	8	4	2
Concentration of haemoglobin in mg%	191	114	62 8	37 4	18 6	10 1
Haemolysed B red cells						
Dilution	1 8	1 16	1 32	1 64	1 128	1 256
Highest dilution capable of inhibiting anti B completely	16	2	2	2	1	0
Concentration of haemoglobin in mg %	221	140	74 3	40 8	21 1	11 2

dilutions of the haemolysates were prepared, and the inhibitory action of these dilutions on anti A and anti-B (4 agglutinating units) was studied

The concentrated solutions of the haemolysates exerted a vigorous inhibitory action on the antibodies. This inhibition could also be demon

Serological Problems

An abnormally high concentration of blood group substance may thus originate from substance-rich secretions, haemolysis and contamination with substance-containing media The resultant serological problems consist in all these cases in difficulties encountered in blood grouping and compatibility tests

The problems associated with blood grouping are due to the fact that the test sera are either partially or completely neutralized by the blood-group substance

In the first three cases, the reactions on Eldon cards were questionable when unwashed cells were used, whereas distinct reactions were obtained with washed blood cells However, unwashed blood cells reacted with strong test sera in test tubes, although the reaction was weak in Case 1 The studies performed showed that the strength of the reactions in the presence of large amounts of substance in the serum was influenced by the concentration of the blood-cell suspension employed The reaction with a 15 per cent suspension was inhibited to a greater extent than that with a 5 per cent suspension It must be presumed that this was caused by the higher concentration of substance in the strong than in the weak suspension In blood grouping by means of Eldon cards according to method II, in which a 15 per cent suspension prepared from stabilized blood is used, it must be recommended to centrifuge the blood samples vigorously before the blood cells are pipetted off In this way, most of the plasma will be removed, so that the influence of the substance in the plasma is reduced

In general, it must be recommended not to use whole blood for blood grouping, as is the case when Eldon cards according to method I are employed Here a drop of blood is transferred direct to the card A moderate increase in the concentration of substance may weaken *or* abolish the agglutination

A determination of the serum content of anti-A and anti-B will reveal errors in blood grouping caused by increased levels of substance in the serum Such a determination should therefore always be routinely employed, also when Eldon cards are used

In blood grouping performed on samples of cord blood or blood withdrawn from newborn infants, the results cannot be checked by studying the serum for anti-A and anti-B, which have not yet developed The correct blood group will be revealed only when washed blood cells are used This check up should therefore be made *routinely in blood* grouping in newborn infants and, in particular, on samples of cord blood, which may have been contaminated with *amniotic fluid*, which in secretors contains large amounts of substance

A high concentration of substance in the serum may also interfere with compatibility tests if sufficiently weak suspensions or washed blood cells are not used ABO incompatibility between recipient and

lymphatics into the blood stream It is also possible that the substance may pass directly into the circulation

Next, it seems as if the blood-group substance may originate from malignant tumours involving exocrine glands Two of the patients described in the literature suffered from cancer of the stomach and pancreas, respectively, and in the present series, two patients had cancer of the stomach In the presence of malignant transformation, the substance may be presumed to be capable of forcing its way direct into the blood stream, either by invasion or formation of metastases However, other factors may also have been of importance in our cases Case 1 showed complete biliary obstruction, and the bile contained large amounts of blood-group substance Owing to the obstruction, some of the substance rich bile may have passed into the blood stream

However, the increase in the concentration of substance in the serum may presumably also be due to haemolysis of the blood samples In Case 2, there was some haemolysis of the first blood sample, which contained 100 mg per cent of free haemoglobin in the serum The two samples which were studied later, and in which the concentration of substance was normal, did not show any haemolysis As demonstrated haemolysis may result in the liberation of large amounts of blood-group antigen In Case 2, an additional explanation is possible, since this patient was admitted with signs of gastric perforation This may have resulted in the passage of substance-containing material into the peritoneal cavity, from which it may have entered the blood stream

Healthy individuals may also show increased concentrations of substance in the serum In one of the previously reported cases and in the present Case 3, the subjects were healthy female donors In our Case 3 the increase was of a transient nature, since a normal concentration was observed 4 months later The first sample studied had been stabilized with heparin A study on a similar heparin preparation for blood group substance was negative The heparin must therefore presumably be ruled out as the source of the substance observed in the plasma, whereas it cannot be excluded that the increase may be due to contamination

Abnormally high concentrations of substance may also be found in the serum of cord blood In Cases 4 and 5, the infants were secretors As amniotic fluid contains large amounts of blood group substance from foetuses who are secretors (*Freda* 1958), it is likely that the blood samples had been contaminated with this fluid This assumption is supported by the fact that the serum from a blood sample withdrawn in Case 5 on the second postnatal day did not contain increased amounts of substance

Finally, it should be noted that the amounts of substance revealed in this series cannot be compared from case to case since the studies were performed with different anti-sera in different dilutions

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PATHOGENESIS OF VASCULAR DISEASE CAUSED BY ACUTE RENAL ISCHAEMIA

By

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In a previous paper (*Giese* 1962) it was shown that severe renal ischaemia in rats gives rise to increased vascular permeability as evidenced by manifestations such as tissue oedema, serous effusions and deposits of serum proteins in arterial and arteriolar walls.

Extreme renal ischaemia causes necrosis of the kidney with suppression of excretory function; an outpouring into *the blood or lymph* stream of several substances escaping from anoxic and necrotic kidney cells will occur under these circumstances. One of these substances is probably the enzyme renin.

On this background it would seem of interest to study the effects of renin and angiotensin in animals with experimental kidney insufficiency.

Several studies in this field are available in the literature, but somewhat conflicting results have been reported. *Winternitz et al.* (1940) and *Masson et al.* (1953) found severe necrotizing vascular disease after injection of kidney extracts or renin into nephrectomized dogs. Studying the effects of renin in nephrectomized rats, *Masson et al.* (1955b) found that arteriolar lesions were less severe and frequent than under like conditions in dogs. In further studies on rats (*Masson et al.* 1956) the formation of serous effusions was again reported, but no acute blood vessel disease was found. *Nairn et al.* (1956) found serous effusions after administration of renal extracts or renin to rabbits, guinea pigs and rats, but again no vascular lesions were reported.

The present study aims at showing whether the syndrome resulting from the application of narrow silver clips on both renal arteries in rats can be reproduced by injections of crude kidney extracts, purified renin and synthetic angiotensin into rats with experimental kidney insufficiency. It was felt that if this was the case, there would be a

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donor will not be revealed by major cross matching if the suspension of donor blood cells contains appreciable amounts of substance

The presence of large amounts of substance in the serum is a relatively rare occurrence If the results of blood grouping are checked as just described, and if sufficiently weak suspensions or washed blood cells are used for compatibility tests, errors due to the presence of blood-group substance may be avoided

SUMMARY

Five cases in which increased amounts of specific blood group substance were revealed in the serum are reported Two of the patients suffered from cancer of the stomach, one was a healthy female donor and two were newborn infants

It is suggested that the substance in the serum may originate from substance-containing secretions, haemolysis of the blood samples or contamination with substance-containing media

The importance of checking the results of blood grouping in adults by a study for anti-A and anti-B, and in newborn infants by repeating the determination on washed blood cells is emphasised Only weak blood-cell suspensions or washed blood cells should be used for compatibility tests

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Group B (5 rats) These rats were sham—operated the peritoneum was opened but the kidneys were not touched After the operation they were given 2 × 5 ml of normal saline i p Autopsy 24 hours after operation showed no effusions or tissue oedema sections of pancreas duodenum and small intestine showed no vascular abnormalities in 4 rats In sections from the small intestine of one animal a few PAS positive deposits in the walls of small arteries were found

Group C (10 rats) These rats were bilaterally nephrectomized and given 10 ml of normal saline after the operation either i p or s c

The findings in this control group are listed in Table 1 In no case was found any pancreatic oedema or peritoneal effusion A limited number of PAS positive vascular deposits were found in sections from four of these rats One rat (no 835) had pronounced vascular lesions in the intestines succulence of the coecal mesenterium was seen at the autopsy

2 *Injections of Crude Rat Kidney Extracts into Bilaterally Nephrectomized Rats*

An exact duplication of the syndrome resulting from extreme kidney ischaemia was obtained The animals were dyspnoeic showing at autopsy oedema of the pancreas succulence of the mesenterium and peritoneal and/or pleural effusions It is noteworthy that peritoneal effusions were found only in those cases where the kidney extract was administered by the intraperitoneal route

Also microscopically exactly the same lesions as found after renal ischaemia were encountered PAS positive deposits in arterial and arteriolar walls with disappearance or deformation of nuclei in the media of many of the affected vessels were the salient findings These deposits could be seen in the whole circumference of some vessels in others only a part of the circumference was involved

A summary of pathological manifestations in six animals treated in this way is given in Table 2 it is quite evident that tissue oedema vascular lesions and the volume of effusions are all very much increased in these rats receiving kidney extract when compared to saline treated control animals (Table 1)

3 *Injections of Crude Rat Kidney Extract into Rats with Bilateral Ligation of the Ureters*

It was found of some interest to study the effect of kidney extract in rats with another form of experimental renal insufficiency The dose was 2 × 5 ml i p

Results are given in Table 3 together with the findings in control animals given 2 × 5 ml of normal saline after ligation of the ureters Effusions pancreatic oedema and vascular lesions similar to those encountered in nephrectomized animals were found after the administration of kidney extract

strong probability, that an outpouring of renin from the ischaemic kidneys is the cause of this syndrome.

MATERIAL AND METHODS

Animals Female albino rats, weighing approximately 200 grammes were used

Preparation of crude kidney extracts Rat kidneys were cut into small pieces and homogenized in 4 ml of ice cold saline per gram of kidney tissue in a glass tissue grinder The homogenate was left overnight at 4° C for extraction After centrifugation in the cold (about 12500 × G for 15 20 minutes) the supernatant was dialysed against normal saline at 4° C for 24–48 hours After dialysis and filtration the extract was diluted with normal saline, so that 10 ml of the final extract would correspond to 1 0–1 2 grammes of kidney tissue The extracts were stored at about —15° C

Purified hog renin This preparation was made according to *Haas Lamfrom & Goldblatt* (1954) 1 ml contained 2000 rat units of renin as defined in this institution; the amount of renin extractable from kidneys of normal rats of this strain is between 750 and 2500 of these units per kidney (*Bing 1962*)

Synthetic angiotensin Hypertensin CIBA (Val5 - angiotensin II - amide) was dissolved in normal saline

Intravenous infusions Infusions were given by an automatic infusion machine through a polyethylene catheter placed in the jugular vein under amytal anaesthesia The catheter was firmly secured in place by several sutures attaching the catheter to the skin on the back of the rat A rubber tube was drawn over the catheter to safeguard against the bite of the rat The animals were kept in ordinary cages with no restraint other than that imposed by the attached catheter

Autopsies and histologic technique At autopsy effusions were collected by a pipette and volumes measured Pancreatic oedema was estimated according to a scale ranging from 0 to +++ Tissues were fixed in buffered formalin Sections of pancreas, duodenum (with attached pancreas), small intestine and heart were prepared in all cases, in selected cases other organs were studied as well Periodic acid—Schiff staining was employed as a routine In the histological assessment of vascular lesions an arbitrary scale ranging from 0 to +++ was likewise employed

General remarks The duration of all experiments (except those recorded in section 6) was 19–24 hours During this time no food or water was allowed, this precaution was taken in order to eliminate variations in fluid intake In injection experiments the volume of fluid administered was 10 ml in all cases, the volumes used in infusion experiments are recorded in the appropriate sections

Injections were given either intraperitoneally (i p) or subcutaneously (s c) In some cases the whole volume of fluid was injected subcutaneously (multiple injection sites) immediately after nephrectomy In most cases two injections (i p or s c) were given one (5 ml) at the start of the experiment (*i e* at the time of nephrectomy), the other injection (5 ml) four to six hours later

Operations were performed under ether or amytal anaesthesia

RESULTS

1 *Control Series*

To form a baseline for the evaluation of pathologic manifestations induced by kidney extracts and renin, three groups of control animals were studied

Group A (5 rats) These rats were taken from the animal house without any pretreatment whatsoever They were killed, autopsy was performed, and PAS-stained sections of pancreas, duodenum (with attached pancreas) and small intestine were studied No macroscopic or microscopic abnormalities were found, no effusions, no tissue oedema, no PAS-positive vascular deposits were encountered

TABLE 3

Pathological Manifestations in Ureter Ligated Rats Given i p Injections of Rat Kidney Extract or Saline

Rat no	Agent injected	Effusion volume (ml)		Oedema of pancreas	Occurrence of PAS positive vascular deposits			
		Pleural cavity	Peritoneal cavity		Pancreas	Duodenum (+ pancreas)	Small intestine	Heart
718	Kidney extract	2 0	4 0	++(+)	++	++	++	0
719	Kidney extract	4 4	1 3	+++	++(+)	+	++	0
724	Kidney extract	3 3	6 0	++(+)	(+)	0	0	0
725	Kidney extract	4 0	7 2	+++	++	+++	++	0
721	Saline	0	0	0	0	0	(+)	(+)
722	Saline	0 1	0 1	0	++(+)	+	0	0
731	Saline	1 0	0	0	++	0	0	0
732	Saline	0	0	0	++	+(+)	0	0

TABLE 4

Findings in Nephrectomized Rats Injected with Purified Hog Renin

Rat no	Effusion volume (ml)		*Oedema of pancreas*	Occurrence of PAS-positive vascular deposits			
	Pleural cavity	Peritoneal cavity		Pancreas	Duodenum (+ pancreas)	Intestine	Heart
825	3 3	2 2	++(+)	++	+(+)	+++	0
827	4 5	0 5	++	(+)	+(+)	(+)	0
829	3 0	0	++(+)	++	+(+)	++(+)	0

TABLE 1

Summary of Findings in 10 Rats Injected with 10 ml of Normal Saline after Bilateral Nephrectomy

Rat no	Dose (ml)	Route of administration	Effusion volume (ml)		Oedema of pancreas	Occurrence of PAS positive vascular deposits			
			Pleural cavity	Peritoneal cavity		Pancreas	Duodenum (+ pancreas)	Small intestine	Heart
675	2 × 5	i p	0.4	0	0	0	0	0	0
835	2 × 5	i p	1.5	0	0	0	(+)	++(+)	0
836	2 × 5	i p	0.6	0	0	0	0	+(+)	0
846	2 × 5	i p	0.3	0	0	0	0	0	0
847	2 × 5	i p	0	0	0	0	0	0	0
625	2 × 5	s c	0.2	0	0	0	+(+)	0	0
626	2 × 5	s c	0.5	0	0	0	(+)	0	0
639	2 × 5	s c	0	0	0	0	0	0	0
859	10	s c	1.2	0	0	0	+(+)	0	0
860	10	s c	0.1	0	0	0	0	0	0

TABLE 2

Pathological Manifestations in 6 Nephrectomized Rats Injected with Crude Saline Extracts of Rat Kidneys

Rat no	Dose (ml)	Route of administration	Effusion volume (ml)		Oedema of pancreas	Occurrence of PAS positive vascular deposits			
			Pleural cavity	Peritoneal cavity		Pancreas	Duodenum (+ pancreas)	Small intestine	Heart
661	2 × 5	i p	0.9	1.4	+(+)	++(+)	+++	+++	+
662	2 × 5	i p	3.7	2.7	++(+)	++(+)	+++	0	0
717	2 × 5	i p	2.2	9.4	+++	+++	+++	++(+)	0
837	2 × 5	s c	8.5	0	++	++(+)	(+)	0	0
689	10	s c	6.0	0	++(+)	+(+)	(+)	+	++
694	10	s c	4.2	0	+(+)	++	+	+	0

normal saline to give concentrations of 5, 50, and 100 microgrammes per ml Two levels of total dosage were used 40–50 microgrammes and 400 500 microgrammes

Results are given in Table 5 together with the findings in control rats infused with similar volumes of normal saline

It will be seen, that pancreatic oedema is found very regularly at the high dosage level, whereas the volumes of effusion fluid generally are rather small as compared with the results after administration of kidney extract The vascular lesions are exactly like the lesions encountered after administration of kidney extract and purified hog renin rather pronounced lesions are found in many cases

6 *Short Time (5 Hours) Infusions of Angiotensin into Rats Nephrectomized about 19 Hours Previously*

It was found of interest to study the effect of a massive infusion of angiotensin into animals in which metabolic disturbances caused by kidney insufficiency had already developed at the start of the infusion The rats were nephrectomized the evening before the experiment, at

nsin or Saline into Nephrectomized Rats

Oedema of pancreas	Occurrence of PAS positive vascular deposits				Other findings
	Pancreas	Duodenum (+pancr.)	Intestine	Heart	
++(+)	++	++	++	0	
0	0	0	(+)	0	
0	0	+	+(+)	0	
+++	++	++	++	(+)	
+++	+++	+++	+++	0	Vascular disease in the liver
+(+)	+	++	+	+	Haemorrhage in the diaphragm
+	0	+	+++	0	
+(+)	(+)	++(+)	+++	(+)	
+++	+++	+++	+(+)	0	Subpericardial focal oedema
+(+)	+(+)	++	+++	0	Adrenal necrosis and haemorrhage
++(+)	(+)	0	++	0	Adrenal haemorrhage—necroses in liver
0	0	0	0	0	
0	0	0	0	+	
0	0	(+)	(+)	(+)	
0	0	0	0	0	
0	0	0	0	0	
0	(+)	0	0	0	
				0	

Substantial effusions or tissue oedema were not found in the control group, but rather pronounced PAS—positive vascular deposits were seen in the pancreas in three cases

The kidneys showed signs of damage (haemorrhages, interstitial oedema) as a result of the ligation of the ureters This renal damage must be taken into account in the evaluation of the findings in the saline—injected control group

4 *Injections of Purified Hog Renin into Nephrectomized Rats*

1 ml of hog renin was diluted with 9 ml of normal saline Two intra peritoneal injections (2 × 5 ml) were given

As shown in Table 4, this preparation elicits the same pathological manifestations as crude rat kidney extracts

5 *Infusions of Synthetic Angiotensin into Nephrectomized Rats*

These infusions were given over 20–24 hours Intraperitoneal in fusion was tried in two cases only (rat 819 & 820), in all other cases the infusion was given intravenously Angiotensin was dissolved in

TABL

Results of Continous Infusions of Angio

	Rat no	Concentration of angiotensin (µg ml)	Volume of fluid infused (ml)	Total dose of angiotensin (µg)	Duration of infusion (hr)	Effusion volume (ml)	
						Pleural cavity	Peritoneal cavity
Infusion of angiotensin	742	5	9 0	45	22	1 6	0 2
	743	5	9 9	50	22	0	0 3
	753	5	7 5	38	24	0	0
	764	50	8 0	400	20	0 8	0
	790	50	10 2	510	22½	0 8	0 5
	794	50	9 3	465	21½	0	0 1
	819*	50	9 0	450	20	0 2	0 5
	820*	50	8 5	425	20	0 1	0 1
	761	100	5 7	570	20½	0 5	0
	762	100	5 2	520	21	0 3	0
	763	100	5 1	510	21	4 3	0
Infusion of normal saline	772	0	8 2	0	22	0 2	0
	789	0	10 2	0	22½	0	0
	791	0	9 2	0	23	0	0
	775	0	4 9	0	22	0	0
	778	0	6 1	0	21½	0	0
	795	0	5 3	0	21	0	0

* = intraperitoneal infusion *In all other cases the infusion was given intravenously*

normal saline to give concentrations of 5, 50, and 100 microgrammes per ml Two levels of total dosage were used 40–50 microgrammes and 400–500 microgrammes

Results are given in Table 5 together with the findings in control rats infused with similar volumes of normal saline

It will be seen, that pancreatic oedema is found very regularly at the high dosage level, whereas the volumes of effusion fluid generally are rather small as compared with the results after administration of kidney extract The vascular lesions are exactly like the lesions encountered after administration of kidney extract and purified hog renin, rather pronounced lesions are found in many cases

6 *Short Time (5 Hours) Infusions of Angiotensin into Rats Nephrectomized about 19 Hours Previously*

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sin or Saline into Nephrectomized Rats

Oedema of pancreas	Occurrence of PAS positive vascular deposits				Other findings
	Pancreas	Duodenum (+pancr)	Intest ne	Heart	
++(+)	++	++	++	0	
0	0	0	(+)	0	
0	0	+	+(+)	0	
+++	++	++	++	(+)	
+++	+++	+++	+++	0	Vascular disease in the liver
+(+)	+	++	+	+	Haemorrhage in the diaphragm
+	0	+	+++	0	
+(+)	(+)	++(+)	+++	(+)	
+++	+++	+++	+(+)	0	Subpericardial focal oedema
+(+)	+(+)	++	+++	0	Adrenal necrosis and haemorrhage
++(+)	(+)	0	++	0	Adrenal haemorrhage—necroses in liver
0	0	0	0	0	
0	0	0	0	+	
0	0	(+)	(+)	(+)	
0	0	0	0	0	
0	0	0	0	0	
0	(+)	0	0	0	
				0	

TABLE 6

Results of Short Time (about 5 Hours) Infusions of Angiotensin or Saline into Rats Nephrectomized about 19 Hours Previously

	Rat no	Volume of fluid infused (ml)	Total dose of angiotensin (µg)	Effusion volume (ml)		Oedema of pancreas	Occurrence of PAS positive vascular deposits			
				Pleural cavity	Peritoneal cavity		Pancreas	Duodenum (+ pancreas)	Intestine	Heart
Infusion of angiotensin (100 µg/ml)	704	3.5	350	0.1	2.6	+(+)	0	+	+(+)	++
	710	3.1	310	0	0	0	0	++	0	0
	711	3.0	300	0	0.3	0	0	0	0	+
	712	3.1	310	0.6	0.4	0	+	++	0	0
	713	3.1	310	0.3	0.7	++	++	++	+++	+
Infusion of normal saline	707	3.2	0	0.6	0.4	0	+	0	0	0
	708	3.2	0	0	0	0	0	0	(+)	0

TABLE 7

Effects of Rat Kidney Extracts, Purified Hog Renin and Synthetic Angiotensin in Normal Rats

Rat no	Agent	Duration of experiment (hours)	Occurrence of PAS positive vascular deposits				
			Pancreas	Duodenum (+ pancreas)	Intestine	Heart	Kidney
728	Rat kidney extract	25	0	0	0	0	0
729	Rat kidney extract	25	0	0	++	0	0
828	Purified hog renin	24	0	0	0	0	(+)
830	Purified hog renin	24	0	0	+(+)	0	0
831	Purified hog renin	24	0	0	(+)	0	0
780	Synthetic angiotensin	20½	+	+(+)	++	(+)	0
782	Synthetic angiotensin	20	++	(+)	+++	+++	0
792	Synthetic angiotensin	22	0	0	+++	0	0

that time they were given 10 ml of normal saline subcutaneously The dose was approximately 300 microgrammes of angiotensin dissolved in 3 ml of saline, infused over about 5 hours

It will be seen from Table 6, that only two out of five rats showed pancreatic oedema, one rat (no 713) had moderate to severe vascular lesions in all organs studied microscopically

7 *The Effects of Rat Kidney Extract, Purified Hog Renin and Synthetic Angiotensin in Animals with Intact Kidneys*

Having found kidney extracts, purified hog renin and synthetic angiotensin to be very angiotoxic in animals suffering from kidney insufficiency it was found of interest to study the effects of these agents in normal animals with intact kidneys Doses were administered, which would give rise to vascular lesions in a nephrectomized rat

2 rats were given 2 × 5 ml of rat kidney extract i p , 3 rats were injected with 2 × 5 ml (i p) of a dilution of purified hog renin made up as described in section 4 3 rats were infused over 20–22 hours with angiotensin (50 microgrammes per ml, total dose about 450 microgrammes)

Significant effusions were not found in any of these rats, and only one (rat 780) showed oedema of the pancreas and then only in a part of the organ

By comparing Table 7 with Tables 2 and 4 it will be seen, that the effect of kidney extract and renin on the vessels is considerably decreased in the presence of intact kidneys Still minimal to moderate vascular lesions are found in the intestines in some of these rats

The three rats infused with angiotensin all showed vascular lesions, which in two of these rats were found in several organs

DISCUSSION

The application of narrow silver clips on both renal arteries in rats elicits in 24 hours a syndrome characterized by increased endothelial permeability (*Giese* 1962) The salient features of this syndrome are serous effusions oedema of the tissues and deposition of serum proteins in the vascular walls

The pathologic manifestations induced by the administration of crude kidney extracts purified renin and synthetic angiotensin to nephrectomized rats are essentially similar to the lesions induced by severe kidney ischaemia Serous effusions and tissue succulence are regularly encountered after administration of these agents to nephrectomized rats although quantitative differences are found depending on the choise of agent and the route of administration The characteristic vascular lesion found after 24 hours of renal ischaemia, *i e* a deposition in the walls of arterioles and small arteries of a substance giving a strong periodic acid—Schiff reaction most often combined

TABLE 6

Results of Short Time (about 5 Hours) Infusions of Angiotensin or Saline into Rats Nephrectomized about 19 Hours Previously

	Rat no	Volume of fluid infused (ml)	Total dose of angiotensin (μg)	Effusion volume (ml)		Oedema of pancreas	Occurrence of PAS positive vascular deposits			
				Pleural cavity	Peritoneal cavity		Pancreas	Duodenum (+ pancreas)	Intestine	Heart
Infusion of angiotensin (100 μg/ml)	704	3.5	350	0.1	2.6	+(+)	0	+	+(+)	++
	710	3.1	310	0	0	0	0	++	0	0
	711	3.0	300	0	0.3	0	0	0	0	+
	712	3.1	310	0.6	0.4	0	+	++	0	0
	713	3.1	310	0.1	0.7	++	++	++	+++	+
Infusion of normal saline	707	3.2	0	0.6	0.4	0	+	0	0	0
	708	3.2	0	0	0	0	0	0	(+)	0

TABLE 7

Effects of Rat Kidney Extracts, Purified Hog Renin and Synthetic Angiotensin in Normal Rats

Rat no	Agent	Duration of experiment (hours)	Occurrence of PAS positive vascular deposits				
			Pancreas	Duodenum (+ pancreas)	Intestine	Heart	Kidney
728	Rat kidney extract	25	0	0	0	0	0
729	Rat kidney extract	25	0	0	++	0	0
828	Purified hog renin	24	0	0	0	0	(+)
830	Purified hog renin	24	0	0	+(+)	0	0
831	Purified hog renin	24	0	0	(+)	0	0
780	Synthetic angiotensin	20½	+	+(+)	++	(+)	0
782	Synthetic angiotensin	20	++	(+)	+++	+++	0
792	Synthetic angiotensin	22	0	0	+++	0	0

SUMMARY

(1) It is shown that the administration of crude rat kidney extracts purified hog renin or synthetic angiotensin to nephrectomized rats elicits a syndrome involving the formation of serous effusions, tissue oedema and PAS positive deposits in arterial and arteriolar walls

(2) This syndrome is essentially the same as the syndrome resulting from severe renal ischaemia in rats (*Giese* 1962)

(3) It is concluded that a combination of kidney insufficiency and an outpouring of renin from the ischaemic kidneys is responsible for the development of increased endothelial permeability

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with a loss or deformation of nuclei in the affected part of the media, is indistinguishable from the vascular lesions induced by administration of kidney extracts, purified renin or synthetic angiotensin to nephrectomized rats

Together, these studies point to the conclusion, that the necrotizing vascular disease and the increased capillary permeability induced by severe kidney ischaemia are caused by the simultaneous occurrence of renal insufficiency and an outpouring of renin from the ischaemic kidneys, with consecutive formation of angiotensin

Since the most prominent feature of the syndrome seems to be a general increase in endothelial permeability, it is of some interest that renin and angiotensin have been shown to induce increased capillary permeability in other experimental situations (*Addis et al* 1949 *Paldino & Hyman* 1954), besides, these two substances are of course potent pressor agents The relationship between the pressor effect and the permeability—increasing effect of these agents is not clear, but sub pressor doses of renin and angiotensin will increase the rate of disappearance of a dye-protein complex from the blood-stream (*Paldino & Hyman* 1954) and proteinuria after intramuscular injections of renin in rats may occur without any measurable increase in the blood pressure (*Sellers et al* 1952)

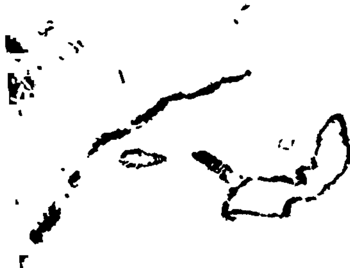
The studies reported in the present paper show, that the effects of renin and kidney extracts are much more pronounced in nephrectomized rats than in normal rats One possible explanation for the increased sensibility to renin in nephrectomized rats might be, that an abnormally increased formation of angiotensin takes place after injection of a dose of renin It is known, that the pressor response to a single injection of renin is abnormally pronounced and prolonged after bilateral nephrectomy (*Blaquier et al* 1962) Rats pretreated with sodium chloride and DOCA exhibit a similarly increased pressor response to a standard dose of renin (*Masson et al* 1955a), renin is very angiotoxic after pretreatment in this way (*Masson et al* 1952)

In the present study three intact rats, infused with a large dose of angiotensin, showed rather pronounced vascular lesions Conclusions concerning a possible protective effect of intact kidneys would in this case demand a much larger material

ADDENDUM

During the completion of the investigations reported in the present paper similar studies made by *Asscher & Anson* (Nature 1963 in press) came to my knowledge through personal communication Their findings concerning the effects of saline kidney extracts administered to nephrectomized rats are in complete accordance with mine anasarca and vascular lesions in heart adrenals and pancreas being found Besides they have shown that the increased endothelial permeability after injections of kidney extract into nephrectomized rats is accompanied by a very considerable decrease of plasma volume They have further shown that the same syndrome can develop after administration of kidney extracts to renal hypertensive rats No effects were found when kidney extracts were administered to normal animals





F gs 1 2

F g 1 A fluorescence microscop cal vie v of a unstained frozen section Magn f ca t on 200 ×

F g 2 The same sect on as in Fig 1 after sta ning w th toluid ne blue Both f gures show that the fluorescence is located in the vascular walls (middle and right) in the l asal meml rane and the m ddle portion of the mucosa Magn f cation 200 ×

FROM THE DEPARTMENT OF PATHOLOGICAL ANATOMY (HEAD PROF OSMO JARVI, M D), UNIVERSITY OF TURKU, TURKU, FINLAND

THE LOCALIZATION OF TETRACYCLINE IN THE METASTATIC CALCIFICATIONS IN THE STOMACH OF RAT INDUCED BY OVERDOSAGE OF DIHYDROTACHYSTEROL AND VITAMIN D_3

By

ILKKA HÄKKINEN and ILMARI LINDGREN

Received 9 V 63

Tetracycline, owing to its strong yellow fluorescence, is easily detected in tissues It has been observed that tetracycline combines itself with malignant growths (*Rall et al* 1957) and especially with the mitochondrias (*DuBuy & Showarce* 1961) The localization of tetracycline in bone tissue is very stable, lasting for weeks (*Mich et al* 1957) However, a more intense fixation is found in the growing new bone than the old (*Harris et al* 1962) *Hakkinen* observed (1958) that tetracycline combines itself with the metastatic calcifications produced by dihydrotachysterol in rats, and DOCA was found to intensify the reaction, but cortisone, on the other hand, again had an opposite effect (1959)

The purpose of this study is to determine the histological localization of tetracycline in the metastatic calcifications in the stomach wall induced by dihydrotachysterol, vitamin D_3 and parathyroid extract The ordinary histological technique is supplemented by fluorescence microscopy and ultra-soft microradiography

MATERIAL AND METHODS

Female albino rats aged 3 months and weighing about 150 g were used During the experiment the animals were given ordinary laboratory food and water The animals were divided into following groups

Group 1 8 animals These were first given 10 mg of dihydrotachysterol (AT 10 Merck) through an oral tube and further 3 mg on the following day The rats were given a single injection of tetracycline (Aureomycin intravenous Lederle), 50 mg/kg body weight Two of the rats were sacrificed on the 3rd 5th 8th and 10th days after treatment

Group 2 (controls) 4 animals These were given an injection of tetracycline as above The animals were sacrificed at the same times as those in group 1 one at a time

Aided by *Sigrid Juselius Foundation*
One of the authors (I L) had the opportunity of studying the biophysical research methods at the department of Pathology II Uppsala Sweden We are greatly indebted to Professor *Bengt Engfeldt* M D for all the facilities he placed at our disposal





Figs 1 2

Fig 1 A fluorescence microscopical view of a unstained frozen section Magnification 200 ×

Fig 2 The same section as in Fig 1 after staining with toluidine blue Both figures show that the fluorescence is located in the vascular walls (middle and right) in the basal membrane and the middle portion of the mucosa Magnification 200 ×

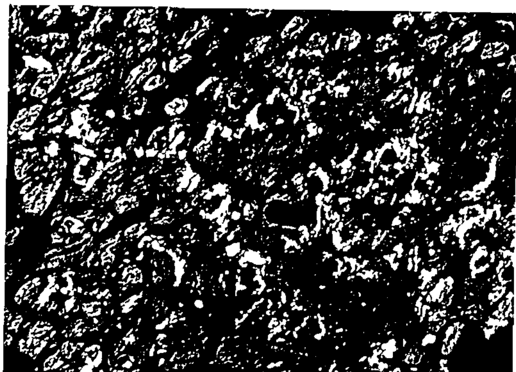


Fig 3

Microradiogram of 5 µ thick horizontal section from the midd portion of *mucosa* The glands are encircled by extracellular calcium salts Exposed at 8 20 Å Magnification 350 ×

Group 3 8 animals These animals were treated exactly as animals in group 1 but AT 10 was replaced by 6 mg of vitamin D_3 (D_3 Vigantol *Bayer*) *which* was given on each of the two days

Group 4 (controls) 4 animals which were treated as group 2 animals and used as control with group 3

Two rats received an intramuscular injection of parathyroid extract (Para Thor Mone Lilly) A dose of 50 IU was given for two days and an injection of tetra cycline *like* all animals in the series These rats were sacrificed after *3* days

When the animals were killed the organs were examined in ultraviolet light (wavelength 3600 A) At first frozen sections were made from fluorescent areas of *the stomach wall and studied by fluorescence microscopy and light microscopy after* toluidine blue staining It was noted however that the ordinary technique for histological staining (formol fixation alcohol xylol paraffin embedding) did not *destroy the fluorescence* and most sections could be treated *in this way*

Fluorescence microscopy was used for unstained and deparafinized sections Ultra soft microradiography was used to study the calcium deposits at cellular level Sections of 5 µ thickness were exposed to x rays in the region of 8 20 Å (*Engstrom* 1956) After the sections had been studied by the methods mentioned above they were stained with haematoxylin eosin haematoxylin Van Gieson periodic acid *Schiff technique and mucicarmin*

RESULTS

When the organs were gross examined it was observed that there was a strong yellow fluorescence in the stomach lungs and kidneys of the animals treated with AT 10 vitamin D or parthyroid extract, plus tetracycline The control animals which had received only tetra



Fig 4

Microradiogramm of a 5 μ thick section from the gastric wall Calcifications are present in the midd portion of mucosa in the basal membrane and in the muscular coat (arrow) Exposed at 8–20 Å Magnification 100 ×

cycline did not present any fluorescence in the organs mentioned However all the animals were noted to have a strong fluorescence in their bones

Fluorescence microscopy showed that the fluorescence was typically located in the stomach wall There was no difference in the groups treated with AT 10 vitamin D_3 or parathyroid extract It is apparent that these three substances have an action of similar nature on the stomach The results will be given in the following considering only the length of the experiment

Findings after 3 days The fluorescence was observed in the basal membrane of the mucosa in the walls of the blood vessels in the muscular coat and a little in the middle portion of the mucosa (Figs 1 and 2) Microscopy showed that these areas contained patches which were stained with haematoxylin The intensity of staining reaction showed a strong variance Microradiography showed that these pathological changes had a great density and were located extracellularly in the fibres of connective or muscular tissue (Fig 3) The walls of the blood vessels did not show any abnormal density

Findings after 5 days The fluorescence had much increased and was located in the same areas mentioned above The haematoxylin staining

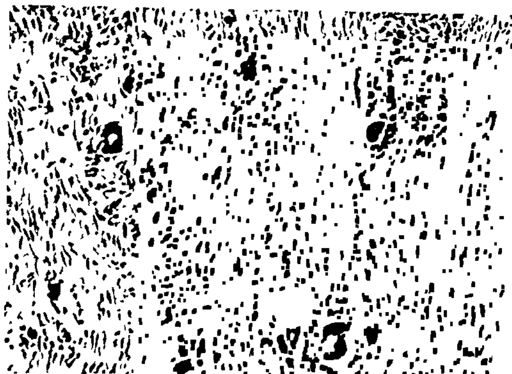


Fig 5
Multinucleated giant cells in a calcified focus of the muscular coat 10 days after the treatment Magnification 400 ×

reaction had increased also In the muscular tissue these areas were infiltrated with macrophages Microradiography showed absorption variations in the same areas (Fig 4)

Findings after 8 days The fluorescence in the middle portion of mucosa had decreased, but remained unchanged in the other areas Histological findings resembled the changes after 5 days The macrophage infiltrations in the muscular tissue had increased, and there were some multinuclear giant cells also Microradiography showed that the abnormal densities had remained unchanged except in the mucosa, were they had decreased

Findings after 10 days The fluorescence had diminished in the middle portion of mucosa, but remained unchanged elsewhere The changes in the muscular tissue showed now plenty of multinucleated giant cells of foreign body type Microradiography showed unchanged conditions

DISCUSSION

It is certain that the abnormal densities, observed by micrography in the different parts of the stomach wall, consist of calcium salts The methods used do not permit a more detailed analysis of their chemical structure

The calcified areas were succesfully stained with haematoxylin, but

there was a great variation in the staining reaction. It could be observed that areas showing a rather strong calcification by microradiography, stained very weakly with haematoxylin. This shows that haematoxylin staining is not a very reliable method for studying calcified structures. It is demonstrated that completely decalcified tuberculous foci still stain with haematoxylin (*Lindgren* 1961). It is apparent that haematoxylin does not stain calcium as such but rather the matrix in which calcium salts are apt to deposit.

It was observed that tetracycline was localized in the metastatic calcifications. Chemically it has been shown that calcium and tetracycline produce a complex compound which can be extracted from the tissues (*Kohn* 1961). Thus it is possible to use tetracycline as an intravital stain for calcium salts.

However it was observed that tetracycline was located in the vascular wall which never presented calcification. It is possible that this is due to the change in the ground substance caused by overdosage of parathyroid extract or related substances (*Engel* 1952, *Laskin et al* 1956, *Gaillard* 1957). This change has apparently the ability of absorbing tetracycline also. The hyperfunction of the parathyroid gland or overdosage of related substances cause metastatic calcifications in different organs especially in the organs with high metabolic activity (*Lehr* 1956, *Selye* 1962). The localization has been explained by the assumption that a rapid ionic exchange occurs in these organs which regulate the ionic balance of the organism. Possibilities for the deposition of calcium salts in these areas should be favourable (*Kleinman* 1928).

This study showed that the metastatic calcifications in the stomach wall were located extracellularly in the connective or muscular tissue fibres. The localization in the middle portion of gastric mucosa is interesting and it is possible that this in some way is connected with the production of hydrochloric acid.

SUMMARY

The overdosage of dihydrotachysterol (AT 10), vitamine D_3 and parathyroid extract caused metastatic calcifications in the gastric wall of the albino rat. Tetracycline given parenterally fixated in these calcifications. This was shown by combined histological and fluorescence microscopical technique and by ultrasoft microradiograpsy. It is concluded that tetracycline can be used as an intravital staining for calcium salts. It is pointed out that tetracycline did also fixate in the changed ground substance of the vascular walls which were not calcified. The metastatic calcifications were located extracellularly in the fibres of muscular and connective tissue.

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A HISTOLOGICAL COMPARISON OF THE EFFECTS OF PREDNISONE, PHENYLBUTAZONE, AND SODIUM SALICYLATE ON GRANULATION TISSUE FORMATION IN OPEN WOUNDS

By

OLE JORGENSEN

Received 16 ii 63

As shown by *Carrel* (3) and later by others (*e g Edwards et al* (7)) the initial inflammatory reaction is of great importance to the normal course of the regenerating processes By treatment with antirheumatic compounds (glucocorticoid hormones, phenylbutazone, and sodium salicylate) *Jorgensen* (11) found the amount of granulation tissue in open wounds to be reduced This reduction was principally due to a decrease of the relative water content of the tissues

The purpose of the present investigation is to describe the histological changes taking place in granulation tissue of open wounds during treatment with prednisone, phenylbutazone, and sodium salicylate

MATERIAL AND METHODS

Female rats weighing on an average 100 g were used

Schedules of treatment

All the drugs mentioned were dissolved in 0 5 ml of physiological saline and injected daily subcutaneously into the occipital region The daily doses were for phenylbutazone 80 mg/kg for sodium salicylate 200 mg/kg for prednisone 5 mg/kg The control animals were g[illegible]ical saline in the same way as the [illegible]ed 5 days before surgical interven[illegible]

Formation of [illegible] by *Rudas* (21) was employed Under ether anaesthesia a circular piece of skin was excised in the midline and distally on the back of the rat The diameter of the ring was larger than that of the hole to keep the ring fixed in the skin

4 or 8 days after the surgical intervention the animals were killed and the granulation tissue dissected out The tissue was fixed in 4 per cent formaldehyde

This investigation was aided by a *grant from the Danish Leage against Rheumatism*

The author is grateful to dr V *Faber* M D the Danish State Serum Institute for gifts of preparations of streptococcal hyaluronidase

Mrs *Dorrit Löt hoft* is thanked for excellent technical assistance

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The following staining techniques have been used

ieson Hansen's method for collagen 3) The
l) Hale's reaction–*Mowry*'s (15) modification
(16) (Staining for 2 hours with a 1 per cent solution at pH 2 7), 6) Azur (0 25 per cent dissolved in Mcllvaine s buffer pH 4 5) Sections stained with azur A were dehydrated in acetone instead of ethanol as recommended by *Curran* (5)

Enzyme Incubations

Sections to be stained with PAS Hale or alcian blue were in some instances incubated with testicular hyaluronidase or streptococcal hyaluronidase The sample of streptococcal hyaluronidase was kindly placed at the author's disposal by dr V Faber, of the Danish State Serum Institute, Copenhagen (8) This enzyme is specific to hyaluronic acid (2) The testicular enzyme (Penetrase®) was manufactured by Leo Pharmaceutical Products Copenhagen The incubations were made at pH 7 0 at 37° for 2 hours The concentration of penetrase was 500 I U per ml

Before staining with azur A some of the preparations were incubated with pepsin for 1 hour at 37° The pepsin—at a concentration of 2 mg/ml— was dissolved in 0 02 N HCl (*Pearse* (18))

RESULTS

Below the crusta the wound tissues under investigation consists of an upper layer of fibroblasts and capillaries infiltrated with inflammatory cells and a deeper layer of fibroblasts, collagenous fibrils and capillaries with no infiltration of inflammatory cells This investigation is only concerned with the deeper layer

Fig 1 shows a haematoxylin-eosin stained section of an 8 days old granulation tissue from the control group Great numbers of fibroblasts are seen arranged in long, parallel formations These cells contain a scanty amount of cytoplasm and darkly staining, fusiform nuclei In v Gieson-Hansen stained sections many collagenous fibrils are seen among the cells (Fig 5) The ground substance is abundant and only very weakly coloured in the haematoxylin-eosin stained sections The

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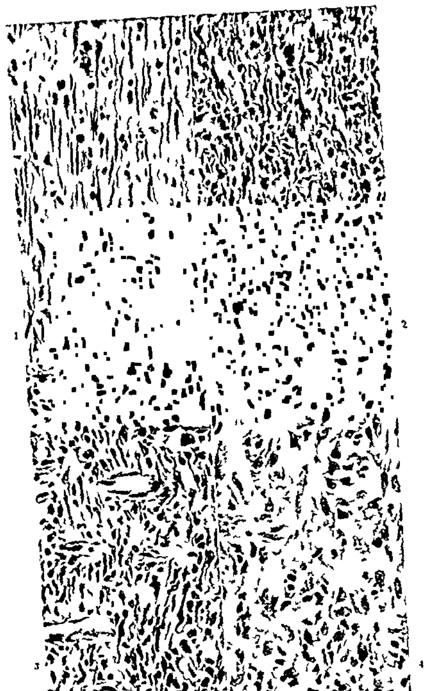
Figs 1–4

Fig 1 Granulation tissue of normal animal Haematoxylin eosin staining Magnification 350 Note long slender fibroblasts resembling fibrocytes Most of the cells are arranged in linear formations The siderophages resembling plasma cells are seen among the fibroblasts

Fig 2 Granulation tissue of prednisone treated animal Haematoxylin eosin staining Magnification 350 Note loss of orientation of the cells and the greatly changed appearance of the individual cells The intercellular substance is considerably reduced in amount

Fig 3 Granulation tissue of animal treated with salicylate Haematoxylin eosin stained preparation Magnification 350 The orderly arrangement is lost some of the fibroblasts contain a lightly stained nucleus others a more darkly staining nucleus The amount of intercellular substance is reduced

Fig 4 Granulation tissue of animal treated with phenylbutazone Haematoxylin eosin stained preparation Magnification 350 The orientation of the fibroblasts is lost The cells are rich in cytoplasm with a vesicular lightly staining nucleus The intercellular substance is reduced in amount



Figs 1 4



Figs 5–6

Fig 5 Granulation tissue of normal animal v Gieson Hansen staining Magnification 350 Many collagenous fibrils are seen among the cells

Fig 6 4 days old granulation tissue of normal animal Haematoxylin eosin staining Magnification 350 The fibroblasts are partly stellate partly spindle shaped and contain large amounts of cytoplasm The intercellular substance is abundant Many young capillaries are seen

ground substance is traversed by many young vessels, mainly capillaries

Treatment with both prednisone, phenylbutazone, and sodium salicylate results in considerable changes of the granulation tissues The total amount of tissue formed is greatly reduced, this effect being most conspicuous in the prednisone treated group Treatment with all compounds results in a reduced amount of intercellular substance which is coloured more intensely in haematoxylin-eosin stained sections than is the intercellular substance of control wounds No alterations are seen, however, in the amount and distribution of capillaries and collagenous fibrils

Great changes are seen in the general arrangement of the fibroblasts of all treated groups The orderly arrangement characteristic of the control wounds is abolished, and in addition the morphology of the in-

dividual cells is greatly changed Many of the cells have acquired a plump appearance with lightly staining, round nuclei, others are more shrunken with a pycnotic nucleus often with indentations, the latter cells being especially numerous in the prednisone treated group Figs 2 3 and 4 represent sections of granulation tissue from animals treated with prednisone, sodium salicylate and phenylbutazone respectively The different appearance of the fibroblasts of Figs 2 and 4 is not real, both types of cells being found in each group The cells of the group treated with prednisone, however, are smaller and lying somewhat more closely than those of the other two treated groups

Fig 6 shows a normal granulation tissue four days of age The large, spindle shaped or stellate fibroblasts lie in an ample amount of intercellular substance with many capillaries In v Gieson-Hansen stained sections delicate collagenous fibrils are seen among the cells It is clearly seen that the fibroblasts of the treated groups do not resemble young cells of normal granulation tissue

Apart from the fibroblasts mentioned another type of cell is found in all groups of granulation tissues In haematoxylin-eosin stained sections these cells often very closely resembles plasma cells with an excentrically placed nucleus and an ample amount of cytoplasm The cytoplasm reacts strongly PAS positive and gives an intense coloration with the Hale method, but does not react with alcian blue or azur A After incubation with hyaluronidase the positive Hale reaction is still seen The coloration is intense too, when the Hale procedure is performed with omission of the colloidal iron reagent This seems to indicate the preformed existence of iron in these cells The cells thus must be [illegible]

[illegible] alcian blue reactions The intensities of both reactions are, however, considerably decreased in the prednisone treated group as compared to the coloration seen in the other groups The PAS reaction is uniformly weak, but definitely positive in all groups, this reaction being most intense in the upper layers of the tissues After treatment with streptococcal hyaluronidase the [illegible] treatment [illegible] completely [illegible] is not influenced by treatment with hyaluronidase The intercellular substance *does not* exhibit metachromasia with azur A When, however, the sections are incubated with pepsin before staining a definite γ metachromasia is seen

Practically no mast cells are seen in the granulation tissue of any group

DISCUSSION

Previous histological investigations have shown that treatment of regenerating tissue with glucocorticoid hormones results in a consider-

able depression of all elements of connective tissue Thus a sparse fibro blast reaction is found both in linear wounds (*Alrich et al* (1), *Pearce et al* (17), *Spain et al* (23)), in carrageenin provoked granulomas (*Fisher & Paar* (9)), and in turpentine abscesses (*Taubenhaus & Amromin* (24), *Zoger* (28)) The fibroblasts are described as immature with an ample amount of cytoplasm and a pale, round nucleus (*Taubenhaus & Amromin* (24), *Zoger* (28)) The nuclei are also described, however, as small and pycnotic (*Taubenhaus et al* (25)) It has also been stated that the amount of capillaries is considerably decreased and that those present often take an abnormal course (*Ragan et al* (20), *Spain et al* (23), *Taubenhaus et al* (25), *Zoger* (28)) A decreased production of collagenous fibrils is also thought to be a characteristic effect of treatment with glucocorticoid hormones (*Alrich et al* (1), *Fisher & Paar* (9), *Pearce et al* (17), *Ragan et al* (20), *Zoger* (28)) This finding is in agreement with biochemical investigations of foreign body granulomas showing a decreased concentration of collagen during treatment with cortisone (*Perkonas et al* (19), *Robertson & Sanborn* (21), *Trnavsky et al* (27)) In granulation tissue from open wounds *Jorgensen* (11) could not confirm this finding and therefore advanced the hypothesis that the somewhat defective collagen formation of foreign body granulomas might be more susceptible to a noxious agent than the normal collagen formation taking place in open wounds The possibility still remained, however, that the total amount of collagen (including soluble precursors) was not influenced by treatment with glucocorticoid hormones whereas the fibrillogenesis might be impaired As is seen from the description of the histological findings, however, prednisone did not influence the formation of collagenous fibrils

The effect of administration of glucocorticoid hormones on the amount of intercellular substance of granulation tissue has been described differently by various authors It has been stated that treatment with cortisone results in a decreased exudation of wounds (*Lattes et al* (14), *Spain et al* (23)) In turpentine abscesses the connective tissue ground substance is both described as loose and oedematous (*Taubenhaus & Amromin* (24)) and reduced in amount As shown here the amount of intercellular substance is considerably reduced both by treatment with prednisone, phenylbutazone, and sodium salicylate

In the present investigation it has not been possible to elicit a positive metachromasia of the ground substance neither in the wounds of normal animals, nor in those of any of the treated groups The fact that treatment of the sections with pepsin before staining with azur A results in a definite γ metachromasia of the ground substance seems to indicate that the blocking effect is due to the presence of a protein in the wound tissue Both *Dunphy et al* (6) and *Taylor & Saunders* (26) have advanced the theory that the metachromasia of wound tissue may be abolished by the presence of protein The latter authors thought that the blocking protein might be collagen this, however is not likely as

a blocking effect of the same nature is also found in granulation tissue of ascorbic acid deficient animals which only contains very little collagen (*Jorgensen* (11, 12))

In the present investigation treatment with prednisone results in a decreased staining intensity of the ground substance both with alcian blue and the Hale reaction. A decreased Hale reaction has also been found by *Pearse et al* (17) in linear wounds of rats treated with cortisone. These authors did not find a concomitant decrease of the metachromatic reaction. Both *Ragan et al* (20), *Spain et al* (24), and *Zoger* (28), however, found the metachromatic reaction to be reduced in intensity during treatment with cortisone.

When the water content of a tissue is reduced, it is reasonable to expect changes to have taken place of the concentration or physical properties of the hyaluronic acid as this mucopolysaccharide is known to be of great importance to the water binding of connective tissue (survey see *Hvidberg* (10)). As treatment with both prednisone, phenylbutazone, and sodium salicylate has been shown to reduce the amount of water in granulation tissue of open wounds (*Jorgensen* (11)) it would have been reasonable to expect the concentration of hyaluronic acid to be reduced by treatment with all 3 compounds. It is possible—indeed—that this is the case but that the biological effect of prednisone in the doses used here is greater than the effect of the other two compounds, so that only treatment with prednisone results in a descernible decrease of the staining reactions. It must be recalled, however, that also chondroitin-sulphate gives a positive staining reaction with the Hale and alcian blue reactions. Thus the possibility must be considered that changes of the concentration of this mucopolysaccharide may cause changes of the staining reactions.

SUMMARY

A comparison of the effects of treatment with prednisone, sodium salicylate and phenylbutazone on the formation of granulation tissue of open wounds has been made. The administration of each greatly changes the morphology of the wound tissue. The orderly arrangement of the fibroblasts is lost, their morphology is changed, and the amount of intercellular substance is reduced in all groups, whereas the intensity of the mucopolysaccharide staining reactions is only reduced by treatment with prednisone.

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FROM THE DEPARTMENT OF PATHOLOGY, MALMÖ ALLMÄNNA SJUKHUS
MALMÖ SWEDEN

PREVALENCE OF THROMBI IN CARCINOMA OF THE PANCREAS

By

J Söderström and L. Bjersing

Received 14 v 63

More than a hundred years ago *Trousseau* (17) noticed the incidence of thrombosis to be relatively high in visceral cancer The frequency of thrombosis in carcinoma, especially carcinoma of the pancreas, has since been the subject of several investigations which have, however, given divergent results (1, 2, 3, 4, 5, 7, 9, 10, 11, 12, 13, 14, 15, 16, 18) Thus it was considered legitimate to re-investigate this problem in an autopsy material

MATERIAL

The material consisted of the autopsy records of 50 consecutive cases of primary carcinoma of the pancreas diagnosed between September 1958 and December 1961 in 27 males and 23 females with an average age of 66 7 years

[illegible]

Control series III was taken from the same period and comprised cases of carcinoma with secondary involvement of the pancreas with the exceptions mentioned above The series included 53 cases but to facilitate compa[illegible] this group was [illegible] were 29 males [illegible]

[illegible] partment which [illegible] tumours and diseases of the vessels and that in all cases vessels are removed from at least the upper half of the thigh and split up

RESULTS

Thrombi were found in the vena cava inferior and/or in the veins of the pelvis or thigh in 26 of the cases of primary carcinoma of the pancreas In 20 cases the thrombosis was bilateral Pulmonary emboli

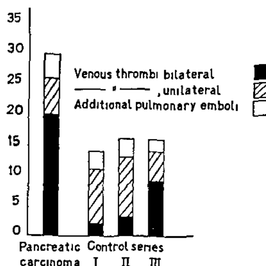


Fig 1

Thrombo embolic disease in pancreatic carcinoma and control groups

were found in 24, including 4 in which no thrombi were observed in any of the above-mentioned vessels

Twenty-three of the patients in control series I had had malignant tumours, but only 19 of them showed residual malignant growth at autopsy Eleven had thrombi in the above-mentioned vessels, but in only 2 of them was the thrombosis bilateral Eleven had pulmonary emboli, including 3 without observed venous thrombi

As to control series II, venous thrombi were found in the vena cava inferior or in veins of the pelvis or thigh in 13 cases The thrombosis was bilateral in 3 cases Pulmonary emboli were noted in 9 cases, including 3 in which no venous thrombi were found

Thrombi were observed in the above-mentioned vessels in 14 cases of control series III The thrombosis was bilateral in 9 Ten cases including 2 without venous thrombi had pulmonary emboli

Thrombosis of the portal vein was not included in the investigation because at least in some cases it might have been due to local tumour infiltration and because the purpose of the study was to ascertain whether pancreatic cancer *per se* tends to increase the frequency of thrombi in general

The 4 series were not found to differ substantially from one another regarding the incidence of thrombi in the aorta pelvic and thigh arteries, coronary vessels, carotid and basal cerebral arteries Nor was any difference noted in the incidence of recent or healed cardiac infarction, mural thrombi of the heart chambers thrombi in the auricles, softening of the brain or status lacunaris

Only 1 of the patients with primary pancreatic carcinoma had a so called terminal endocarditis, a condition seen mainly in association with malignant tumours (5, 6, 10, 12, 13, 14) and said to be common in patients with carcinoma of the pancreas (5, 10 12 13, 14)

DISCUSSION

The incidence of thrombi was found to be substantially higher in the patients with primary carcinoma of the pancreas than in any of the 3 control series. The frequency of thrombi in the vena cava inferior, pelvic and thigh veins was significantly higher ($p < 0.01$ with χ^2 analysis) than that in control series III, which had the highest incidence of thrombi in these vessels of the control series. Our result argues against the view expressed by some authors that thrombi are equally common in secondary and in primary carcinoma of the pancreas (5).

It has often been claimed that thrombi are more common in patients with carcinoma of the body or the tail of the pancreas than in those with carcinoma of the pancreatic head (5, 9, 10, 11, 12, 14). Of our 50 cases the caput was affected in all except 9 cases so that no valid conclusions could be drawn. The findings in the present investigation argued, if anything, for the reverse.

The higher incidence of thrombi in the cases of primary carcinoma of the pancreas might have been due to the patients having been in a poorer general condition, to having had more extensive tumours or having been bedridden for a longer time than the patients in the control series. This possibility seems less likely, especially since the tumour at any rate in control series III appeared to be at least as advanced as in the cases of primary carcinoma of the pancreas.

Each of the groups appeared to be very uniform and all four of them were comparable as to sex distribution and mean age, also regarding control materials II and III which were not selected according to age.

The frequency of thrombo embolism in the control materials was also of the same order as that found in a previous autopsy investigation in Malmö (8). The findings suggested that the higher incidence of thrombi is related to primary and not to secondary carcinoma of the pancreas. This might be due to the production in the primary cancer growth of some factor capable of favouring thrombosis.

Further pathological and clinical investigations of this problem are desirable.

SUMMARY

In an autopsy material of 50 cases of primary carcinoma of the pancreas 26 showed venous thrombi. The frequency was significantly higher than in (a) age matched controls, (b) subjects with extra pancreatic carcinoma and (c) subjects with secondary carcinoma of the pancreas.

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FROM THE DEPARTMENT OF SURGERY, BLOOD BANK AND INSTITUTION OF PATHOLOGY KAROLINSKA SJUKHUSET AND KAROLINSKA INSTITUTET, STOCKHOLM 60 SWEDEN

BLOOD GROUP SPECIFIC SUBSTANCES IN HUMAN GASTRIC CARCINOMA

A Study using the Fluorescent Antibody Technique

By

A.-E. EKLUND, B. GULLBRING and B. LAGERLÖF

Received 14 x 63

Since the report by *Aird, Bentall & Roberts* (1953) of a relationship between blood group A and gastric carcinoma, a large number of papers have been published on the relations between blood groups and both gastric carcinoma and other diseases—for reviews, the reader is referred to *Roberts* (1956 1957, 1957) and *Wiener* (1962) In Sweden, *Beckman & Eklund* (1961) have studied the associations between ABO blood groups and gastric carcinoma in altogether 2 600 patients collected from hospitals in three different regions of Sweden In one of the cancer series there was a highly significant increase of the incidence of blood group A, while in the other two no increase of blood group A was demonstrable on comparison with the control series

Farher studies in this sphere have chiefly utilized genetic and statistical methods on human materials Only in a small number of investigations has the problem been studied at the cellular level The fluorescent antibody technique according to *Coons* (*Coons & Kaplan* 1950) has earl

Holborow & J

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specific substances but used almost exclusively normal tissues

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on the other hand, could demonstrate some antigens even in the sup
ficial mucosal layer of non-secretors They had a single case of gast
carcinoma showing positive fluorescence

Another method of studying blood group antigens is the mixed c
agglutination test originally described by *Coombs & Bedford* (195
According to *Holborow, Brown, Glynn, Hawes, Gresham, O Brien*
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tract contained A and B antigens and that tumours arising from th
epithelium may fail to exhibit these antigens

The present investigation was intended to determine whether tl
blood group specific antigens were lost with tumour formation Gastr
carcinoma was chosen as a suitable experimental material Non-cance
ous gastric mucosa—which is known to contain blood group specif
substances—could be obtained from the same operative specimens ar
hence constituted a good control material

MATERIAL AND METHODS

The series studied comprised 21 gastric tumours—20 adenocarcinomas and o
atypical but benign polyp—and non cancerous mucosa from several of the tumo
patients as well as from patients with peptic ulcers The material is selected On
a few patients belonging to blood group O are included as staining controls Tl
distribution of blood groups in the different specimens in the series will be four
in Table 1 Secretor status had been noted in 18 of the tumour patients and on
two—both belonging to blood group A—were non secretors

TABLE 1

Distribution of Blood Groups in Specimens Investigated

Blood Group	A	B	AB	O
Non cancerous mucosa	11 (1)	3 (1)	5 (1)	4
Polyp		1		
Highly differentiated carcinoma	1			
Moderately differentiated carcinoma	3 (1)			
Low differentiated carcinoma	10 (1)	1	1	4

Figures in parenthesis refer to non secretors

Sera Human blood grouping serum was obtained from immunized single bloo
donors The serum was inactivated at 56° C for 30 minutes Titre examination wa
carried out according to the technique described in the *Minimum Requirement*
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Anti A Titre 1 1 024 1 512 1 128 against A_1 A and A B blood cells respectively
Anti B Titre 1 256 1 128 against B and A_1B blood cells respectively

Anti human globulin serum (AHG) Titre 1/102400 in the passive haemagglutina
tion test with tanned red cells according to *Boyden* (1951)

The AHG serum used was conjugated with fluoresceinisothiocyanate according t

Mellors (1959) proposals After conjugation the serum was absorbed twice w th calf liver powder and stored at —20° C *

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Preparation of Tissue Sections Specimens were collected at operation Tissue from both the carcinoma and normal mucosa was taken at gastrectomy or partial gastric resection Biopsy specimens were excised from glandular metastases and frozen sections prepare l from the greater part of them

on sanatux glass without any fixation The mounted sections were stored at —20° C

Fluorescence Staining Technique The indirekt or two-step method was used

sections were immediately covered with ordinary cover glasses and examined in a fluorescence microscope

Staining Controls Two consecutive serial sections were always prepared and stained simultaneously In the first step anti A and anti B sera respectively were

RESULTS AND DISCUSSION

Normal mucosa from patients belonging to blood groups A, B and AB always showed positive fluorescence In agreement with the findings of *Glynn et al* (1957), this was invariably strongest on the surface of the mucosa and in superficial portions of gastric pits, decreasing in deeper parts of the gastric body In sections from the pyloric region positive fluorescence was also seen close to the pyloric glands Much of this derived from mucus on the surface of the cells, as is illustrated in Fig 2 but distinct fluorescence was also seen in the cellular membrane itself even in the absence of demonstrable mucus (Fig 3)

The distribution of the fluorescent substance was found to be independent of the secretor status For control, four other anti-A sera were used separately with the same result Our result thus differ from those obtained by *Szulman* These differences may be due to the

* The authors are indebted to Professor *Astrid Fagraeus* at Statens Bakteriologiska Laboratorium for kindly providing the serum

the mucus secretion or surface epithelium *Glynn, Holborow & Johnson* on the other hand, could demonstrate some antigens even in the superficial mucosal layer of non-secretors They had a single case of gastric carcinoma showing positive fluorescence

Another method of studying blood group antigens is the mixed cell agglutination test originally described by *Coombs & Bedford* (1955) According to *Holborow, Brown, Glynn, Hawes, Gresham, O'Brien & Coombs* (1960) there is a close correlation between the results obtained with the mixed cell agglutination test and the fluorescent antibody technique *Cowan* (1962) examined nine cases of gastric carcinoma by mixed cell agglutination In the seven cases belonging to blood group A and B he got positive agglutination *Kay* (1957) studied A and B antigens with the same technique in bladder tumours of varying degrees of malignancy He concluded that the epithelium of the human urinary tract contained A and B antigens and that tumours arising from that epithelium may fail to exhibit these antigens

The present investigation was intended to determine whether the blood group specific antigens were lost with tumour formation Gastric carcinoma was chosen as a suitable experimental material Non-cancerous gastric mucosa—which is known to contain blood group specific substances—could be obtained from the same operative specimens and hence constituted a good control material

MATERIAL AND METHODS

The series studied comprised 21 gastric tumours—20 adenocarcinomas and one atypical but benign polyp—and non cancerous mucosa from several of the tumour patients as well as from patients with peptic ulcers The material is selected Only a few patients belonging to blood group O are included as staining controls The distribution of blood groups in the different specimens in the series will be found in Table 1 Secretor status had been noted in 18 of the tumour patients and only two both belonging to blood group A—were non-secretors

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The AHG serum used was conjugated with fluoresceinisothiocyanate according to

staining technique and or differences in the titres of the sera—compare *Coons* (1956), *Jankovic* (1959), *Jankovic & Lincoln* (1959), *Cohen, Zuelzer & Evans* (1960) On the contrary, the findings are in agreement with the results of *Hartmann* (1941) In her comprehensive study she found water-soluble group specific substances produced both by secretors and non secretors, and differences between them being only quantitative

The controls were negative in every instance in which the sections had been stored frozen for a short period, and the control samples belonging to blood group 0 were likewise negative Non specific fluorescence was distinguishable in four cases, in all these instances the interval between sectioning and staining had been long—at least one month Three of these patients belonged to blood group A, the fourth to blood group 0 This nonspecific fluorescence was weak—according to *Mellors* the autofluorescence increases appreciably during storage—and staining following incubation with homologous antibodies demonstrated specific fluorescence unaffected by the storage

All but one of the tumours showed positive fluorescence The exception was a low differentiated scirrhous carcinoma belonging to blood group A In this case, the large amount of connective tissue in the tumour also interfered with the conventional microscopic examination The apparent absence of distinguishable fluorescence might therefore be due to the small number of tumour cells Otherwise, no difference in the intensity of the fluorescence was noted between normal mucosa and tumour tissue As is seen in Figs 4 to 8, occasional cells and small groups of cells also showed fluorescence, and this was apparently not dependent upon the demonstrable presence of mucus A carcinoma producing moderate amounts of mucus exhibited fluorescence wholly corresponding to that of the other carcinomas Nor did the tumours from the two cases which were non secretors differ demonstrably in fluorescence from the secretors

The fluorescent antibody method is best suited for qualitative investigations and no attempt has been made at quantitative estimation of the content of different group antigens

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Figs 3–8 (please turn over)

Fig 3 Fluorescent non-cancerous mucosa from the gastric body

Fig 4 Fluorescent gland in a [illegible]

Fig 5 Rather highly *differenti*[illegible] developed mucus-secreti[illegible]

Fig 6 Positive fluorescence in [illegible] carcinoma

Fig 7 Fluorescent cancer cell groups in a metastasis from a low differentiated adenocarcinoma

Fig 8 Dissociated cancer cells in a low differentiated carcinoma of the scirrhous type Very distinct fluorescence is seen in the malignant cells

Figs 3 8 are all from secretors belonging to blood group A All sections have been treated with anti A serum and AHG Magnification × 500



Fig 1

Non cancerous gastric mucosa blood group O treated with anti A serum and AHG Only a week autofluorescence can be seen Magnification × 500 Exposure time 30 minutes

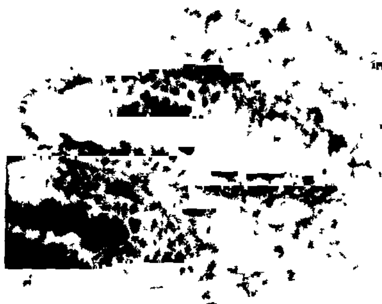


Fig 2

Non cancerous gastric mucosa Blood group A non secretor Section treated with anti A serum and AHG Strong positive fluorescence in mucus and cell membranes together with a week autofluorescence Magnification × 500 Exposure time 4 minutes

staining technique and/or differences in the titres of the sera—compare *Coons* (1956), *Jankovic* (1959), *Jankovic & Lincoln* (1959), *Cohen, Zuelzer & Evans* (1960). On the contrary, the findings are in agreement with the results of *Hartmann* (1941). In her comprehensive study she found water soluble group specific substances produced both by secretors and non secretors, and differences between them being only quantitative.

The controls were negative in every instance in which the sections had been stored frozen for a short period, and the control samples belonging to blood group 0 were likewise negative. Non specific fluorescence was distinguishable in four cases, in all these instances the interval between sectioning and staining had been long—at least one month. Three of these patients belonged to blood group A, the fourth to blood group 0. This nonspecific fluorescence was weak—according to *Mellors* the autofluorescence increases appreciably during storage—and staining following incubation with homologous antibodies demonstrated specific fluorescence unaffected by the storage.

All but one of the tumours showed positive fluorescence. The exception was a low differentiated scirrhous carcinoma belonging to blood group A. In this case, the large amount of connective tissue in the tumour also interfered with the conventional microscopic examination. The apparent absence of distinguishable fluorescence might therefore be due to the small number of tumour cells. Otherwise, no difference in the intensity of the fluorescence was noted between normal mucosa and tumour tissue. As is seen in Figs. 4 to 8, occasional cells and small groups of cells also showed fluorescence, and this was apparently not dependent upon the demonstrable presence of mucus. A carcinoma producing moderate amounts of mucus exhibited fluorescence wholly corresponding to that of the other carcinomas. Nor did the tumours from the two cases which were non secretors differ demonstrably in fluorescence from the secretors.

The fluorescent antibody method is best suited for qualitative investigations and no attempt has been made at quantitative estimation of the content of different group antigens.

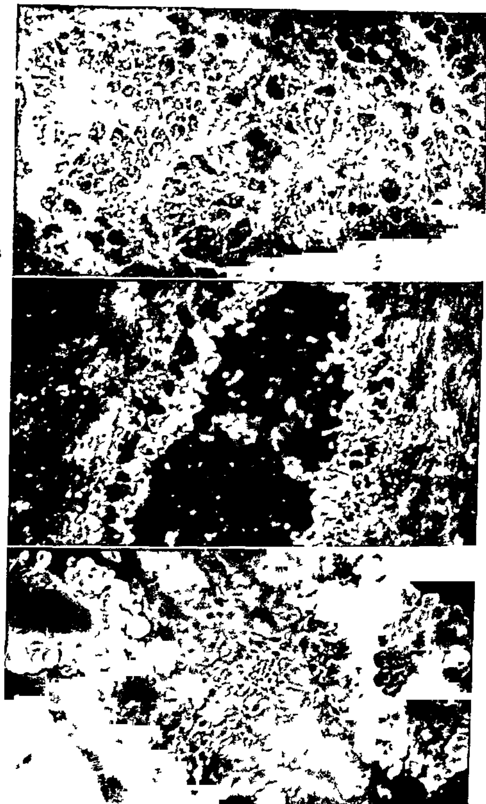
Figs. 3–8 (please turn over)

Fig. 3. Fluorescent non cancerous mucosa from the gastric body.
Fig. 4. *Fluorescent gland in a highly differentiated adenocarcinoma.*
Fig. 5. Rather highly differentiated adenocarcinoma. Positive fluorescence in well developed mucus secreting glands and in groups of cancer cells in the stroma.
Fig. 6. Positive fluorescence in solid cell groups from a low differentiated adenocarcinoma.
Fig. 7. Fluorescent cancer cell groups in a metastasis from a [illegible] differentiated [illegible]
Fig. [illegible] the scirrhous

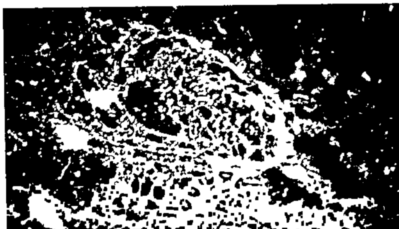
Figs. 3–8 are all from secretors belonging to blood group A. All sections have been treated with anti A serum and AHG. Magnification × 500.

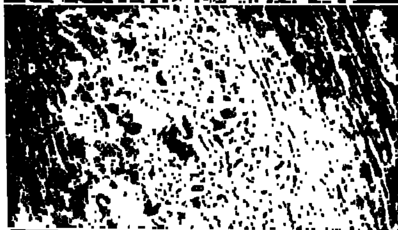
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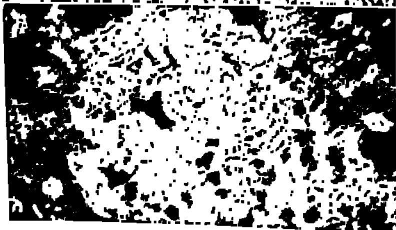
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Figs 3 5


6


7


8

Figs 6-8

With the technique used it was, then, possible to detect blood group specific substances in both normal gastric mucosa and in neoplasms developing in it. The degree of differentiation of a human gastric carcinoma does not seem to affect the occurrence of blood group specific substances in the tumour cells themselves—as determined qualitatively with the technique used by us.

SUMMARY

The fluorescent antibody technique has been used in fresh tissue sections to study the occurrence of blood group specific substances in normal gastric mucosa and gastric carcinomas of varying degrees of differentiation. In normal mucosa fluorescence was localized to mucus both in glands and on the mucosal surface, as well as to the cellular surface itself. The results were not dependent on the secretor status. With one exception—a scirrhous carcinoma poor in cells—all the carcinomas also showed positive fluorescence.

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TUBERCULOSIS IMMUNIZATION RESEARCH CENTRE STATENS SERUMINSTITUT COPENHAGEN DENMARK AND SCHWEIZERISCHES FORSCHUNGSINSTITUT DAVOS PLATZ SCHWEIZ

SEROLOGICAL CHANGES IN PRI MICE CARRYING OVARIAN TUMOUR PASSAGES

By

J M RHODES and AUDREY FJELDE

Received 18 x 63

The sera of mice carrying certain tumours and plasma cell leukemias have been investigated electrophoretically by various authors (*Potter et al* 1957 1959 *Rask Nielsen et al* 1959 *Clausen et al* 1959) Their findings suggest that a number of changes occur in the serum globulins in these diseases in accordance with the changes in the serum patterns reported in multiple myeloma in human beings

Clausen et al (1959) have applied the technique of immunoelectro phoresis to this problem and demonstrated that the β and γ globulins are not only altered in mice carrying transplantable plasma cell leu kaemia but also in experimental amyloidosis and transplantable mouse hepatomas

The present paper describes the application of the same techniques in an investigation of the sera of mice carrying an ovarian tumour

MATERIAL AND METHODS

Animals

The strains of PRI mice used for these experiments were obtained from Dr Char lotte Friend The Sloan Kettering Institute New York (1954) and Dr Albert Sabin Childrens Hospital Cincinnatti (1958) The granulosa cell tumours of the ovary were found in mice after injection of an aqueous extract of a skin tumour (*Fjelde* 1958 1961 *Fjelde & Sorkin* 1960) The skin tumour was received from Dr Charlotte Friend The Sloan Kettering Institute New York 1954

For the first passage of the ovarian tumours studied a mince was made of the frozen material and 0 2 ml was injected into the lateral abdominal surface of 20 PRI mice 3 5 months old (1st passage group 1)

A mince of the same frozen ovarian tumours was also injected into 18 PRI mice 3 4 months old (1st passage group 2)

Blood was obtained from mouse 49 in the first group of animals by heart punc ture and from No 83 from the ruptured spleen In the second group of animals blood was obtained by *heart puncture 3 4 weeks after injection*

The normal control mice were of the same stock and approximately the same age as the tumour bearing mice

Total protein was determined by the method of *Lowry* (19[illegible]2) 10 μl of each serum was diluted to 1 ml and 100 μl of these dilutions was used for each determination Tyrosine 50 mg per cent was taken as the standard (*[illegible] et al* 1956) The colour reactions were measured *in a Beckman* spectrophotometer *D U* at *750 mμ*

Paper Electrophoretic Technique

A horizontal apparatus was employed[1] 5 µl of the sera (10 µl for No 83) was applied to Schleicher & Schull paper strips Electrophoresis was carried out for 19 hours at 200 V man 8mA in veronal buffer pH 8 6 (μ = 0 1) at room temperature The strips were stained for protein with Amidoschwarz 10 B, for lipid with Sudan Black (*Swahn* 1952) and for carbohydrate with Schiff's reagent (*Hirsch & Cattaneo* 1957)

The paper strips stained for protein were evaluated in a Spinco analytrol and the figures obtained for the areas of the peaks were used for calculating the relative per cent of the proteins in the sera

Immunoelectrophoresis

The technique used was a modification of Scheidegger's micromethod which is fully described by *Heremans et al* (1959) The stains employed were identical with those mentioned under paper electrophoresis

Antiserum

g 3 rabbits a total of ter the last oled The γ The preci-

pitated γ globulin was dissolved in sufficient water to give a 6 times concentrated solution This solution was then dialysed to phosphate buffer pH 8 6 and the final dialysate was employed in the immunoelectrophoretic technique

RESULTS

The Transplant

The induced tumours grew to twice the volume of the host and were extremely well vascularized, showing no degeneration after periods of 9 10 months of growth No tumours were induced in Group II after inoculation of the frozen minced tumours

Histology

Description of the Ovarian Tumour

Invasively growing tumour tissue, composed of rounded, solid formations of closely packed cells, occasionally, a folliculoid pattern could be seen, or a cylindrical appearance could be encountered The cells were relatively small, uniform with rather sparse [illegible] eosinophilic cytoplasm, and rather [illegible] nuclei Many mitoses were seen Sta[illegible] negative The tumour type was difficult to establish, but it appeared to be a granulosa-cell tumour of the ovary Fig 1 is a photograph of the original ovarian tumour

Pathological Findings

The two mice which accepted the transplant of tumour cells showed the following pathology

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[1] Supplied by LKB Produkter Fabriksaktiebolag Stockholm Sweden

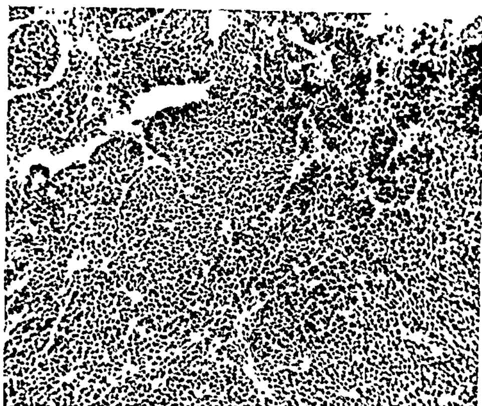


Fig 1
Photograph of ovarian tumour

Mouse 49[2] *(Group I, 1st Passage)*

Macroscopic —The mouse (male) showed a large tumour twice the volume of the host The mouse was well-nourished and the tumour showed no necrosis and was well vascularized The spleen showed a considerable amount of haemorrhage and in rupturing discharged a large amount of blood into the abdominal cavity Death was due to haemorrhage following splenic rupture (hypervolaemia)

Microscopic —The following pathological changes were seen —Tumour cell metastases to the lung necrosis, and dilated sinusoids in the liver, extra medullary haematopoiesis in sinusoids, many pycnotic nuclei in the liver cells, and amyloid deposits were seen

Mouse 83

Macroscopic —Same as above
Microscopic —Same as above

Serological Changes Shown in Paper Electrophoresis

Paper electrophoretic determinations of the sera from normal mice and mice inoculated with tumour mince are shown in Table 1

[2] We are indebted to Dr *Jorgen Ringsted* pathologist Bispebjerg Hospital Copenhagen, Denmark, for the descriptions of the mouse tumours

Table 1

Paper Electrophoretic Determinations

Normal PRI serum

Albumin	48.5 per cent	(minium 45.0 per cent	maximum 52.5 per cent)
α_1 globulin	4.7 „	„ 2.4 „	6.9 „
α_2 globulin	16.6	3.4	18.8 „
β globulin	19.2	„ 18.2 „	19.8
γ globulin	10.4 „	„ 9.2	12.2 „

Total amount of protein 6.08 gm percent (min 5.2 gm percent max 6.7 gm percent)

Serum No 49

Albumin	35.7 per cent	(minium 30.5 per cent	maximum 40.0 per cent)
α_1 globulin	4.0	„ 2.2	5.7
α globulin	14.3 „	12.0	16.8
β globulin	38.9	„ 36.5	40.6
γ globulin	7.0 „	„ 5.6	„ 9.8 „

Total amount of protein 6.03 gm percent (min 6.01 gm percent max 6.06 gm percent)

Serum No 83

Albumin	32.8 per cent	(minium 30.5 per cent	maximum 36.0 per cent)
α_1 globulin	3.7	2.8	4.9
α_2 globulin	3.5	6.2	„ 13.6 „
β globulin	38.8 „	„ 28.8	„ 48.0 „
γ_2 globulin	15.1 „	„ 6.7 „	20.7 „

Total amount of protein 3.1 gm percent (min 2.5 gm percent max 3.7 gm percent)

The total protein of the pathological sera lies within the normal range but it is evident from the table that there is an increase in the β globulins to the detriment of the albumin component and in some cases the γ globulin component

Figs 2–4 illustrate conventionally stained paper electrophoretic analysis of normal and pathological PRI mouse sera from the first passage of the ovarian tumour

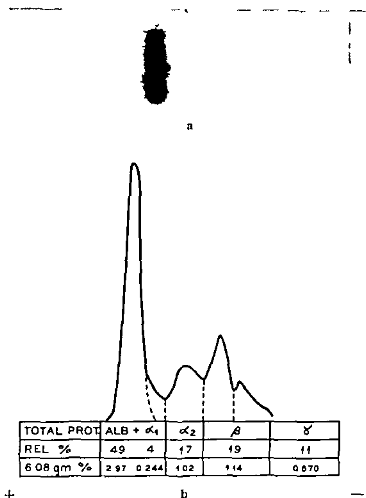
The β globulin in the serum from the first passage of the ovarian tumour (No 49 Group I) is greatly increased from 1.4 g per cent to 2.35 g per cent total protein Correspondingly, the albumin fraction is reduced from 2.97 g per cent to 2.17 g per cent total protein

Lipid staining of these sera after electrophoresis is diffuse although there appears to be a peak in the α fraction Carbohydrate staining shows the presence of sugars in the β, α and γ globulins with increase of carbohydrate in the β globulin fraction

Immunoelectrophoresis

Fig 5 illustrates a conventionally stained agar electrophoresis of normal PRI serum and No 49 Group I It is evident that there is a decided increase in the β protein with corresponding decrease in the γ globulin and the albumin in the pathological sera

These two facts were borne out by immunoelectrophoresis As can



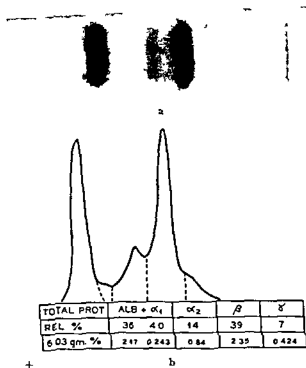
TOTAL PROT	ALB + α_1	α_2	β	γ
REL %	49 4	17	19	11
6 08 gm %	2 97 0 244	1 02	1 14	0 670

Fig 2

a) Conventional paper strip electrophoresis of normal PRI serum
b) Scanning diagram of a)

be seen in Fig 6, there are several changes in the serum pattern of No 49 1) The γ globulin bow which normally extends far into the β zone is much shorter and stops in front of the normal γ globulin line which is *indicative of hypo-γ-globulinaemia 2) There is an increase* in the β_2-I bow This protein has been shown to be identical with transferrin in human serum (*Clausen et al* 1960b) 3) There is a strong increase in the α_2 V bow Carbohydrate staining showed it to be a glycoprotein 4) β_2 III is increased slightly in concentration it can be distinguished in the pathological serum and not in the normal serum, presumably due to the fact that our antiserum did not contain a sufficient quantity of antibodies to this protein to enable it to be visualized in the normal serum

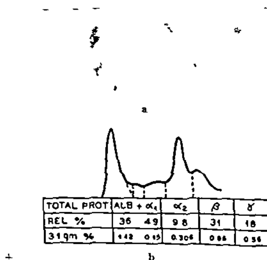
Lipid staining of both pathological and normal sera indicated only one main lipo-protein, namely α -I



TOTAL PROT	ALB + α_1	α_2	β	γ
REL %	36 40	14	39	7
6 03 gm. %	2 17 0 243	0 84	2 35	0 424

Fig 3

a) Conventional paper strip electrophoresis of pathological serum No 49
b) Scanning diagram of a)



TOTAL PROT	ALB + α_1	α_2	β	γ
REL %	36 49	9 8	31	18
3 1 gm %	1 42 0 15	0.306	0 96	0 56

Fig 4

a) Conventional paper strip electrophoresis of pathological serum No 83
b) Scanning diagram of a)

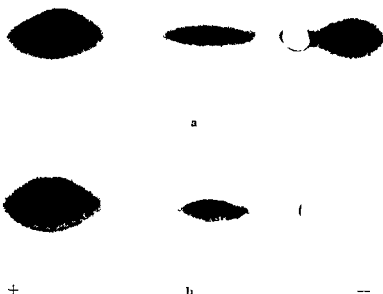


Fig 5

Conventionally stained agar electrophoresis of normal PRI serum
Conventionally stained agar electrophoresis of pathological serum No 49

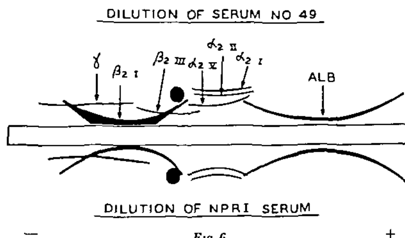


Fig 6

Schematic drawing of immunoelectrophoresis of pathological serum No 49 and normal PRI serum both diluted 1/4

DISCUSSION

A transplantable granulosa cell tumour of the ovary associated with leukaemia and hypervolaemia was induced in mice (AK and RF) by *Furth & Sobel* (1946) Their findings appear to be related to those described here

Examination of the sera of PRI mice carrying an ovarian tumour in the first passage (Group I) showed that there was hypo γ globulin aemia an increase in the β-III protein in the β-I protein and in the

case of the 49 serum, also in the α_2-V protein Sera from mice inoculated with the frozen tumour mince (1st passage, Group II) in the majority of cases showed the same type of changes as described above, despite the fact that no tumours were induced in this group

A tumour was also induced in one animal from another group of mice which were injected with the same frozen ovarian tumour The serum from this animal showed the same characteristic changes in the protein pattern as those in Group I

An increase in the α_2-V globulin has been demonstrated in nephritis in mice (*Clausen et al* 1960a) There is an indication that this protein may be similar to the α_2 macroglobulin in human serum described by *Schultze* (1957)

The increase of β I and β_2-III proteins in some pathological conditions has already been demonstrated by *Clausen et al* (1959, 1960) in plasma cell leukaemia, experimental amyloidosis, and in hepatomas in mice The increase in β_2-I is somewhat peculiar since transferrin (identical to β_2-I in mice) in human beings is usually reduced in most diseases in man Thus, transferrin is diminished in multiple myelomatosis (*Schultze et al* 1957)

It should also be mentioned that *Clausen et al* (1960) have observed increases in β globulins in mice inoculated with pulmonary carcinoma and mice injected with human albumin This increase occurs notably in β_2-III which these authors have termed immune globulin

It is not clear whether these increases, apart from the increase in α_2-V, are the outcome of a generalized cell proliferation in response to the antigens of the tumour or whether it is a non-specific stimulation resulting in increased globulin production

Recent work carried out in a preliminary chromosome study of one ovarian tumour indicated that this had a normal stem line of 40 A specific chromosome pattern was found for each type of 4 different plasma cell leukaemias, producing respectively, γ globulin, β 2 A para protein, amyloidosis of the kidney, and amyloidosis of the kidney, spleen and liver (*Fjelde et al* 1962) The significance of the chromosome pattern, which seems to develop in passage, is not understood, nor is the relationship of the abnormal chromosomes to the abnormal protein produced However, such a system of tumour cells might be useful in studying the *in vitro* production of protein from subcellular particles

SUMMARY

The pathology of an ovarian tumour *in the first* passage is described Changes in the sera of mice carrying this ovarian tumour as investigated by paper electrophoresis and immunoelectrophoresis are described There was a large increase in β_2-I, β_2-III and α_2-V globulins

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FROM STATENS SERUMINSTITUT, COPENHAGEN, DENMARK
THE STREPTOCOCCAL DEPARTMENT (CHIEF KNUD SKADHAUGE, M.D.)

INFECTION AND MALIGNANT TUMOURS

6. *The Heat Stability of the Tumour-Inhibiting Factor in Extract from Haemolytic Streptococci, and a Comparison of Extracts from a Streptococcal, a Staphylococcal and a Coli Strain in their Effect on Brown-Pearce Carcinoma*

By

EBBE AHRENSBURG CHRISTENSEN

Received 21.v.63

In a previous study (6) it was demonstrated that a cell-free extract from mechanically ground haemolytic streptococci could inhibit Brown-Pearce carcinoma in young rabbits.

In the present study the heat stability of the tumour-inhibiting factor in such a streptococcal extract was examined, and the inhibitory effect of the extract was compared with the effect of corresponding extracts from a strain of Staphylococcus aureus (209-P) and a strain of Escherichia coli (D 279).

The historical background for the interest in the antagonism between bacterial infections and malignant tumours, and a review of the most important experimental studies, can be found both in a number of older publications (2, 14, 1, 12) and in more recent ones (9, 3, 5).

MATERIAL AND METHODS

Young male rabbits of a white Danish breed ("Copenhagen Whites" from Hvidesten, the breeding farm of Statens Seruminstitut) were used in the experiments. All the animals were 8 or 9 days old at time of [illegible] were performe[illegible] Pearce carcino[illegible] treatment wi[illegible] The treatment [illegible] of intraperitoneal injections the f[illegible] af[illegible] w[illegible] of [illegible] the animals was judged on their increase in weight and on their appearance. All animals were weighed on the day of transplantation, three days later, subsequently once a week and finally [illegible] [illegible] [illegible] investigator using a standard technique. On the basis of the autopsy findings the animals were classified in three groups according

to the occurrence of metastases (A) and in three groups according to the quantity of tumour tissue (B) (7)

The extracts were prepared as follows

Twenty four hour cultures of the bacteria in trypsin bouillon were centrifuged for 20 minutes at 3000 r p m The wet bacterial mass was ground with an equal volume of alumina and the mixture extracted three times with isotonic phosphate buffer saline, pH 7 38, for 24 hours each time All procedures were carried out at a temperature between 0° and 8° C (6) Extracts were prepared from the following bacterial strains

Streptococcus pyogenes group A, type 12 no 3465
Staphylococcus aureus (FDA 209-P)
Escherichia coli (D 279) (from The International Escherichia Centre Statens Seruminstitut, Copenhagen)
Isotonic phosphate buffer saline pH 7 38, was used as placebo

RESULTS

In the *first series of experiments,* an examination was made of the heat sensitivity of the streptococcal extract The extract was heated on a water-bath to 50° C, 60° C and 100° C, respectively, for 30 minutes The tumour-inhibiting effect of the heat-treated preparations was compared with the effect of untreated extract

A total of 113 animals were used, their distribution into the various treatment groups being shown in Fig 1 Three animals died before the 12th day after transplantation, i e before the tumour had developed sufficiently, and had therefore to be excluded from the comparisons Two of these animals were being treated with unheated extract, while the third had been given extract heated to 50° C Of the remaining 110 animals, 30 had received unheated extract, 32 placebo, and 16, 20 and 12 extract heated to 50° C, 60° C and 100° C, respectively

Heating to 100° C resulted in complete disappearance of the tumour-inhibiting effect of the extract After heating to 60° C, the extract had no longer any effect on the metastasization, while there seemed to be some inhibition of the local tumour growth in the peritoneum, as relatively many of the animals had only a small amount of tumour tissue at autopsy, even though metastases were found The result of heating to 50° C for 30 minutes was that the extract lost some of its tumour-inhibiting effect, but the material was not extensive enough to decide just how much was lost

In the *second series of experiments,* the extract from haemolytic streptococci was compared with extracts from *Staphylococcus aureus* and *Escherichia coli,* respectively

A total of 111 animals were employed, the distribution of these in the various treatment groups being indicated in Figs 2 & 3 A total of 37 animals received extract from streptococci, 37 placebo, 22 extract from staphylococci and 15 extract from E coli

Neither the extract from the staphylococci (Fig 2) nor from the coli (Fig 3) had any effect on the metastasization However, both extracts seemed to inhibit growth of tumour in the peritoneum in a

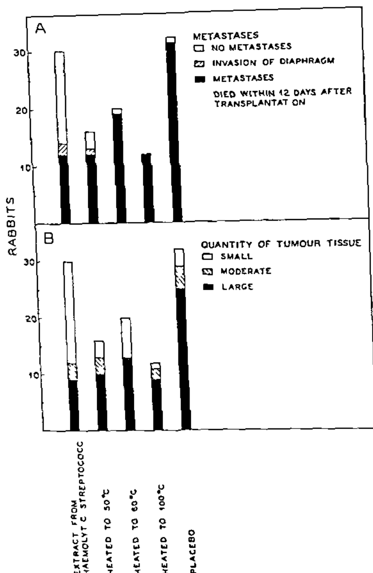


Fig 1 A and B
Metastas zati n and quantity of tumour tissue in experiments with heat treated extract fr m *Streptococcus pyogenes*

number of the animals although this inhibition did not occur quite so frequently as among animals receiving streptococcal extract Corresponding doses of streptococcal extract gave pronounced inhibition of both metastasization and tumour growth in the peritoneum in about half of the animals

to the occurrence of metastases (A) and in three groups according to the quantity of tumour tissue (B) (7)

The extracts were prepared as follows

Twenty four hour cultures of the bacteria in trypsin bouillon were centrifuged for 20 minutes at 3000 r p m The wet bacterial mass was ground with an equal volume of alumina and the mixture extracted three times with isotonic phosphate buffer saline, pH 7 38, for 24 hours each time All procedures were carried out at a temperature between 0° and 8° C (6) Extracts were prepared from the following bacterial strains

Streptococcus pyogenes group A type 12 no 3465
Staphylococcus aureus (FDA 209 P)
Escherichia coli (D 279) (from The International Escherichia Centre Statens Seruminstitut, Copenhagen)
Isotonic phosphate buffer saline pH 7 38, was used as placebo

RESULTS

In the *first series of experiments*, an examination was made of the heat sensitivity of the streptococcal extract The extract was heated on a water-bath to 50° C, 60° C and 100° C, respectively, for 30 minutes The tumour-inhibiting effect of the heat-treated preparations was compared with the effect of untreated extract

A total of 113 animals were used, their distribution into the various treatment groups being shown in Fig 1 Three animals died before the 12th day after transplantation, i e before the tumour had developed sufficiently, and had therefore to be excluded from the comparisons Two of these animals were being treated with unheated extract, while the third had been given extract heated to 50° C Of the remaining 110 animals, 30 had received unheated extract, 32 placebo, and 16, 20 and 12 extract heated to 50° C, 60° C and 100° C, respectively

Heating to 100° C resulted in complete disappearance of the tumour-inhibiting effect of the extract After heating to 60° C, the extract had no longer any effect on the metastasization, while there seemed to be some inhibition of the local tumour growth in the peritoneum, as relatively many of the animals had only a small amount of tumour tissue at autopsy, even though metastases were found The result of heating to 50° C for 30 minutes was that the extract lost some of its tumour-inhibiting effect, but the material was not extensive enough to decide just how much was lost

In the *second series of experiments*, the extract from haemolytic streptococci was compared with extracts from *Staphylococcus aureus* and *Escherichia coli*, respectively

A total of 111 animals were employed, the distribution of these in the various treatment groups being indicated in Figs 2 & 3 A total of 37 animals received extract from streptococci, 37 placebo 22 extract from staphylococci and 15 extract from E coli

Neither the extract from the staphylococci (Fig 2) nor from the coli (Fig 3) had any effect on the metastasization However, both extracts seemed to inhibit growth of tumour in the peritoneum in a

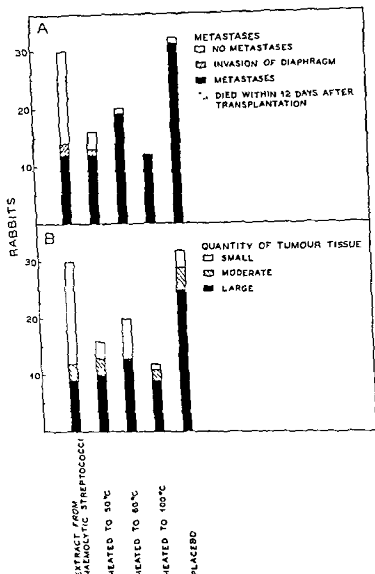


Fig 1 A and B

Metastasization and quantity of tumour tissue in experiments with heat treated extract from *Streptococcus pyogenes*

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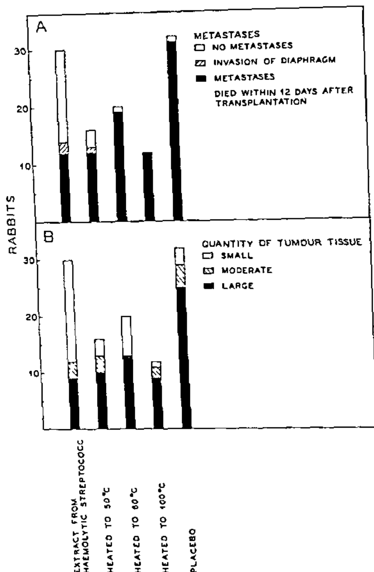


Fig. 1 A and B

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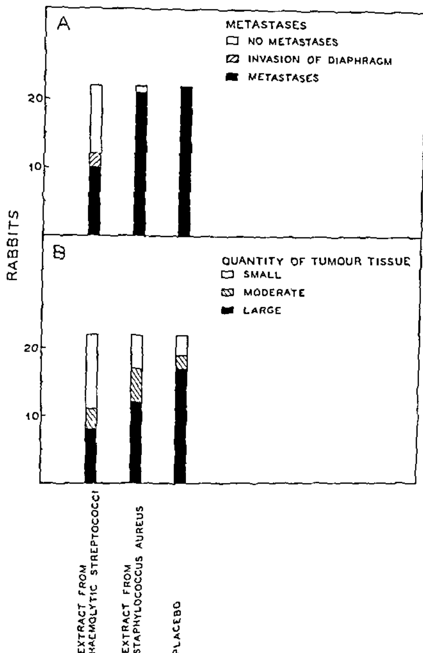


Fig. 2 A and B

Metastasization and quantity of tumour tissue in experiments with extracts from *Streptococcus pyogenes* and *Staphylococcus aureus*

DISCUSSION

In previous experiments with haemolytic streptococci, it was occasionally observed that a preparation could be completely without effect on Brown-Pearce carcinoma, and that a preparation which had had a tumour-inhibiting effect shortly after being prepared, might be ineffective later. It has been shown (4) that bacterial contamination of

coccal and coli extract while the metastasization was unaffected by both these preparations *Goldie et al* (8) were able to demonstrate a local tumour inhibiting effect of infections with Gram negative bacteria while heat inactivated organisms were without effect This need not contradict the result in the experiments described here in which the local tumour inhibition was produced by cell free extracts In performing the extraction allowance was made for the fact that a tumour inhibiting factor could be thermolabile and in addition the treatment was so intensive that the amount of bacterial endotoxins and endo enzymes administered to each animal may be regarded as being of the same order of magnitude as in infection Finally the experiments were performed in rabbits while *Goldie et al* used mice

However the effect of the staphylococcal and coli extracts on Brown Pearce carcinoma could be clearly distinguished from that of streptococcal extract In the present series of experiments as well as in all former experiments where streptococcal lysates or extracts were used in young rabbits inoculated with Brown Pearce carcinoma the active preparations inhibited both the metastasization and the local growth of tumour in the peritoneum The only exception has been the experiment already described where extract was heated to 60° C for 30 minutes It was previously demonstrated (4) that the effect of the streptococcal preparations on Brown Pearce carcinoma appears to be independent of the vascularization of the tumour tissue In contrast to this the local inhibition of tumour which was demonstrated by *Goldie et al* in the case of infections with Gram negative rods and which was also observed in the present investigation after treatment with extracts of staphylococci and coli seems to operate only in the case of cells in the ascites fluid while cells which are nourished by circulating blood are not inhibited

SUMMARY

A cell free extract of haemolytic streptococci which inhibited *metastasization* and tumour growth in peritoneum in about 50 per cent of young rabbits with Brown Pearce tumour transplanted to the peritoneum lost part of its inhibitory effect when heated to 50° C for 30 minutes After heating to 60° C the extract had no effect on *metastasization* while there was still some inhibition of the tumour growth in the peritoneum After heating to 100° C the extract had no effect on the tumour

Extracts of Staphylococcus aureus and B coli had no effect on the metastasization but both extracts had a moderate inhibitory effect on the tumour growth in the peritoneum

effect could be due to temperature sensitivity of the tumour-inhibiting factor

Preliminary attempts to determine the temperature sensitivity of the factor in lysates of streptococci produced with the aid of streptococcal bacteriophages gave *contradictory* results *A possible explanation* of this may be that the lysates contained too many substances unessential in this connection However, with extracts from mechanically disrupted streptococci it was possible to eliminate not only extraneous substances from the culture substrate, but also exotoxins and exo enzymes Extracts of this kind were used in the present experiments, which as mentioned showed rapid destruction of the tumour-inhibiting factor between 50° and 60° C This agrees with the observation by *Koshimura & Shoin* (11) that heating to 56° C for 30 minutes inactivated an extract of streptococci which, before heating, had been able to reduce the take percentage of Ehrlich ascites carcinoma when the cells were incubated together with the extract prior to transplantation

With extracts from mechanically disrupted streptococci it was possible also to compare the tumour-inhibiting effect of these with the effect of corresponding extracts from other bacteria To provide adequate information as to any possible tumour-inhibiting effect of the two bacterial strains investigated, the present experiments should also have included tests with such large doses of extract that the limit of toxicity was reached, just as extracts of the bacteria should have been tested after a longer period of growth and in other media This was not done because such investigations can be performed more conveniently in an easier assay-system *Koshimura et al* (10, 11) used for example an *in vitro/in vivo* technique in which Ehrlich ascites carcinoma cells were incubated for 90 minutes at 37° C together with bacteria or a cell-free preparation from these The percentage of takes and the survival time after transplantation of the mixture to mice were then used as a measure of the tumour-inhibition Using this technique, a total of nine strains of haemolytic streptococci belonging to group A and 10 strains from other species (pneumococci, staphylococci, gonococci, and other species) were examined for their tumour-inhibiting effects (10 13) Ability to prevent the take of Ehrlich carcinoma was demonstrated only in group A streptococci Among the nine strains tested, *three were very effective in inhibiting the tumour*, three were moderately effective and two had a very slight effect while one strain had no effect whatever

The experiments with staphylococcal and coli extracts in the present study must also be considered in the light of the well-known phenomenon that infections with virus or bacteria may cause failure of both normal and neoplastic tissue transplantation, and that some infections can reduce the number of ascites fluid tumour cells in ascites tumours (8) In the experiments described here, a local influence on the tumour cells in the peritoneal fluid was clearly present, both with staphylo-

FROM THE DEPARTMENT OF PATHOLOGY II (PROF. S RANSTRÖM)
UNIVERSITY OF GOTHENBURG, SWEDEN

SUCTION TECHNIQUE FOR SEDIMENTATION OF BODY FLUID CELLS

By

SVERKER ENESTRÖM

Received 10 vi 63

Few body fluids are rich enough in cells for direct cytologic examination. They must first be concentrated in some way. Occasionally the fluid itself solves this problem by depositing a coagulum, rich in cells. Other fluids can be made to do so artificially. Routine methods for separating cells from the fluid are mechanical and include such processes as centrifugation or filtration. The literature abounds with recommendations of and variations on concentration procedures designed to improve the cell yield. Among common technical variations, it will suffice to mention the use of specially constructed centrifuges (*e g* Spiral and Threshold centrifuges (4, 16), direct centrifugation onto slides (6, 13, 14), pretreatment of the sample with various additives (fixation, precipitation or suspension agents, separating, haemolyzing or mucolytic substances, etc), repeated centrifugation, and combinations of centrifugation and filtration.

Unfortunately cell sedimentation with the aid of centrifugal force is attended by varying degrees of cell injury, a problem that has attracted particular attention in the study of the cytology of cerebrospinal fluid (C S F) (9 20). C.S.F. cells have often remained inadequately preserved despite numerous modifications of the centrifugation technique (7 18 20) and filtration through a Millipore membrane has failed to produce wholly satisfactory results.

New means of attaining improved cell preservation have been provided by the advent of simple sedimentation technique which, moreover, simplifies the actual
concentration procedure. Rather than [illegible]
let the cells sediment directly on a [illegible]
preservation the procedure require [illegible]
unless the sample has a high cell [illegible]
creasing the sedimentation rate an [illegible]
sediment. Sayk tried to drive the [illegible]
utilizing the capillary absorption [illegible] us materials. Optimum flow velo-
city was obtained when filter paper was used as the "motor" (19). A [illegible]
incorporating this [illegible]
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tion chamber [illegible]
fluid content [illegible] from the lower perimeter of the [illegible] side and its sedimentation chamber

This work was supported by a grant from the *Medical Faculty of Gothenburg*

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New means of attaining improved cell preservation have been provided by the advent of simple sedimentation technique which moreover, simplifies the [illegible]

[illegible] materials Optimum flow velocity was obtained when filter paper was used as the "motor" (19) A construction incorporating this principle is diagrammed in Fig 1 The sedimentation chamber proper is fitted into a piece of thick walled rubber tubing [illegible]
stands on a [illegible]
paper is [illegible]
tion chamb[illegible]
fluid conter[illegible] [illegible] perimeter of the sedimentation chamber

This work was supported by a grant from the *Medical Faculty of Gothenburg*

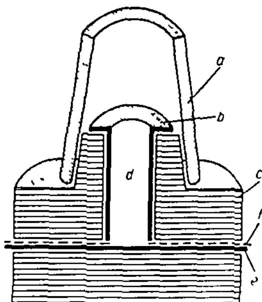


Fig 1

The Sayk sedimentation chamber (Illustration reproduced from *Sayk J Cytologie* der Cerebrospinalflussigkeit Jena, Gustav Fischer, 1960 by kind permission of Professor J Sayk)

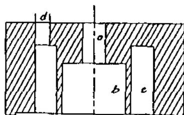
at a rate proportional to the pressure of the rubber tube on the filter paper, the pressure being adjusted by means of a weight sliding on a lever

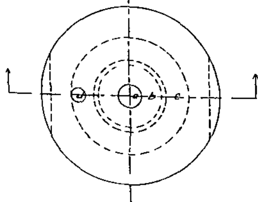
The cells are not exposed to any significant strain as they slowly are propelled towards the slide where they are deposited while the fluid escapes into the filter paper At the recommended pressure of 3 kg on the filter paper 1 ml of CSF generally takes 20 to 30 minutes to escape from the sedimentation chamber some 50 to 60 per cent of the sample's cell content being recovered The advantages of this method are that the time factor can be controlled that preparation artefacts are reduced and that durable and readily stainable specimens can be obtained with apparatus which is uncomplicated and simple to operate Applications of this sedimentation chamber are discussed in the literature (1 5 19–22 27)

The Sayk sedimentation chamber is suitable only for such samples whose fluid phase readily can pass through the filter paper capillaries Fluids of greater viscosity than CSF tend to sediment at an inconstant rate and the fact that the fluid column in the Sayk chamber is not visible makes it difficult to adjust the flow velocity When the velocity is excessive a significant proportion of the cells will be lost by adhesion to the edge of the paper or entry into it After the sample's cell content has been concentrated in the Sayk sedimentation chamber the fluid phase cannot be recovered Hence when only small samples are available (as often applies in the case of CSF) a difficult balance must be struck as to how the available volume shall be divided between cytologic examinations and chemical analyses

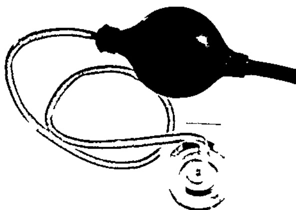
THE SUCTION CHAMBER

Attempts to eliminate the disadvantages of the Sayk sedimentation chamber without losing any of its advantages proved unsuccessful It became evident that the motive power for vertical displacement of the fluid column had to be something other than capillary attraction In order to enable recovery of the fluid phase and ensure a smoothly adjustable flow system, it was decided to utilize a simple differential pres-





A



B

Fig 2

A Construction of suction chamber a roof aperture b inner chamber, c outer chamber, d suction aperture
B Suction chamber on slide and utilizing a simple suction balloon, *for ambulant use*

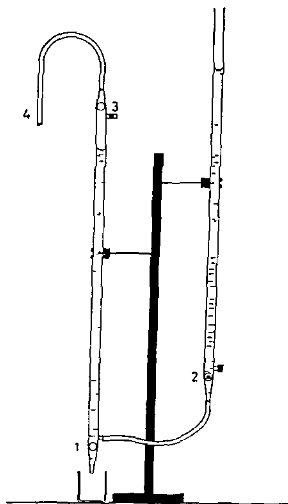


Fig 3

Suction device 1 suction burette stopcock, 2 pressure burette stopcock, 3 shunt valve, 4 tube to suction aperture

sure method As appears from the functional diagram in Fig 2, the solution was to combine two chambers one connected to a suction device, *the other under atmospheric pressure The two chambers are* coaxial and the rim of the outer extends beyond that of the inner, leaving a capillary space The double chamber is placed on a slide and sedimentation occurs, predominantly on the slide surface in the inner chamber, as the fluid column is slowly transferred to the outer chamber, when its pressure is *reduced* This is accomplished by a suction burette in series with a pressure burette (Fig 3) When the stopcock of the suction burette is opened, the pressure in the outer chamber diminishes in proportion to the volume of liquid removed from this burette This volume can be read off on the burette graduations and is equal to the volume of sample transferred from the inner to the outer chamber The transfer rate can be accurately controlled and changed at any time By opening the stopcock of the pressure burette the fluid



Fig 5
Cerebrospinal fluid A neutrophils and a basophil (meningitis) (× 1536), B, tumour cells with a pseudo rosette (malignant plexus papilloma no primary tumor found elsewhere in the body) (× 950)

can be returned to the inner chamber for resedimentation Cells that have escaped into the outer chamber will then be deposited there as an external ring Made of plexiglass, the suction chamber enables the sedimentation and resedimentation processes to be directly inspected The sedimented cells will never be exposed to pressures exceeding the initial hydrostatic pressure (i e the specific gravity of the sample multiplied by the height of the fluid column in the inner chamber) After

sedimentation of the cells from the sample, its fluid phase remains available for chemical analysis

If a voluminous sample must be examined in its entirety, the inner chamber can be filled repeatedly through its roof aperture and the outer chamber may be drained via a T-tube in the line to the suction burette A shunt valve enables the suction burette to be replenished from the pressure burette without upsetting the pressure relationships in the system But very voluminous samples are more simply handled by using Millipore membrane according to *Seal* (23)

The suction chamber has been tried on a large number of samples of various fluids, including C S F, pleural fluid, ascites fluid, urine and cystic puncture fluid The method was originally intended for studies of C S F cytology and most slides were fixed and stained according to Pappenheim (first May-Grunwald solution and then Giemsa solution) (12), also recommended for body fluids other than C S F (2, 8, 25), although considerable advantages accrue from the use of Papanicolaou's OG-6-EA stains in cytodiagnosis directed towards the detection of tumour cells (10, 11)

The suction chamber technique has a number of outstanding advantages over its predecessors Thus the cell yield is increased by the possibility of resedimentation on the same slide Another means of increasing cell yield is to sediment the same sample on multiple slides The concentration process is very gentle and the collected cells adhere strongly to the glass The cells lie flattened out on the glass surface so that the cellular and nuclear structures become clearly visible, a fact that facilitates accurate cytologic evaluation and differentiation in for instance, the C S F (Fig 4)

DISCUSSION

In clinical practice there is need for a simple and rapid method whereby body fluids can be cytologically examined The cell concentration methods mentioned in the introduction—as well as the cell block technique (17, 28)—are often complicated Cell concentration should neither consume nor destroy the sample's fluid phase, which must remain available for chemical analysis The latter requirement is met by the suction chamber technique which not only enables recovery of the fluid phase but also renders prefixation unnecessary because the sample is examined in the fresh state Although the mutual adhesiveness of malignant cells is poor (3), they attach themselves firmly to glass (15) Whilst leucocytes and lymphocytes are supposed to adhere poorly to glass (15), even these cells are extensively recovered on suction chamber slides If prefixation must be done to avoid autolytic cell injuries formalin-fixed cells attach to a glass surface almost to the same extent as cells in fresh fluids Addition of serum reduces cell adhesiveness to the substratum (25, 26) but, intended for elimina-

tion of cell injuries by centrifugation (7, 18), tends to cause clumping of cells (21) Elongated cells are frequently encountered in the cell fraction of directly centrifuged specimens, in a Millipore membrane filter multiple cell layers are formed if the sample is rich in cells and above a critical pressure the cells are deformed in its pores

SUMMARY

A simple and rapid suction technique for concentrating the cells in a sample of body fluid is described and discussed in the light of other methods

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FROM THE HISTOLOGIC DEPARTMENT OF THE KAROLINSKA INSTITUTET, STOCKHOLM 60 SWEDEN HEAD PROF N A HILLARP

THE POSTNATAL DEVELOPMENT OF THE MITOCHONDRIAL CONTENTS IN BLOOD LYMPHOCYTES OF NORMAL AND OXYGEN-EXPOSED MICE

By

U Ernstrom and B Larsson

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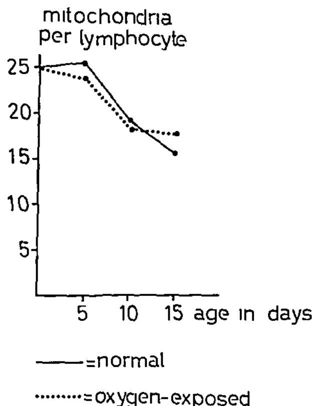
Concentrated oxygen is known to have a noxius effect upon the maturation of newborn animals Thus oxygen exposure interferes with the normal development of retinal and cerebral vessels (*Ashton et al* 1954 *Michealson et al* 1954, *Gyllensten et al* 1954, *Gyllensten* 1959) and with the haematopoiesis (*Campbell* 1928, *Cooperberg et al* 1951, *Gyllensten et al* 1959) The mechanism of the toxic effects of oxygen has been supposed to depend upon interference with the respiratory enzymes (see *Bean et al* 1944 *Stadie et al* 1944), but the relationship has not been established

During the investigations of the influence of thyroxin on the thymolymphatic tissue, the present authors observed an increase of the mitochondrial contents of blood lymphocytes in hyperthyroid guinea pigs (*Ernstrom & Larsson* 1961) Knowing the intimate relationship between mitochondria, cell respiration and oxygen consumption, the authors wanted to examine the effect of continuous exposure to concentrated oxygen on the mitochondrial contents of the blood lymphocytes of newborn animals As a control experiment, the normal postnatal change of the number of mitochondria in the blood lymphocytes was studied

MATERIAL AND METHODS

59 newborn male and female black mice (stock C 57 BL/6) were employed 39 were used as untreated [illegible]

[illegible] technl[illegible]
[illegible] counts we[illegible]
magnifical[illegible]



RFSULTS

The blood lymphocytes of newborn and 5 days old normal mice showed relatively large contents of mitochondria, but from this age on the contents decreased rapidly In the normal mice 10 and 15 days old, the mitochondrial contents of the lymphocytes were only 76 and 63 per cent respectively of the corresponding value of the newborn animals (Fig 1) This decrease was highly significant (p < 0 001)

TABLE 1

Differential Mitochondrial Counts of Lymphocytes in the Blood of Vormal and Oxygen Exposed Newborn Mice

Age in days	Treatment	Number of mice examined	Number of lymphocytes examined	Classification of lymphocytes according to their mitochondrial contents Lymphocytes)) per cent						Average number of mitochondria per lymphocyte
				0-10	11 20	21-30	31-40	41 50	51-60	
0	Controls	11	600	5 0	28 8	38 7	18 8	5 8	2 8	25 0 ± 1 9
5	Controls	11	600	5 5	27 7	38 8	17 3	6 5	4 2	25 3 ± 1 8
5	Oxygen exp	10	500	5 6	32 6	38 6	16 2	5 4	1 6	23 8 ± 2 2
10	Controls	8	400	23 6	31 8	29 5	12 3	2 5	0 3	18 9 ± 3 2
10	Oxygen exp	10	500	18 0	44 0	30 0	6 6	1 0	0 4	18 1 ± 2 1
15	Controls	9	550	30 9	42 7	19 6	4 5	1 8	0 4	15 8 ± 1 2
15	Oxygen exp	10	500	15 4	48 6	30 2	5 8	0 2	0 0	17 7 ± 1 1

The animals exposed to concentrated oxygen from birth showed a retarded general development Thus the animals exposed to oxygen from 15 days had a body weight and a general appearance corresponding to that of normal animals 5–10 days old The mitochondrial contents of the blood lymphocytes of the animals exposed to oxygen, did not, however, significantly differ from the corresponding control values (Table 1)

DISCUSSION

During the first postnatal week the blood lymphocytes of mice have large contents of mitochondria During the second week the contents decreased to 63 per cent of the value at birth The cause of this post natal decrease of the mean mitochondrial number of the circulating lymphocytes is only speculative

Previous investigations have shown a correlation between the mitochondrial contents of the circulating lymphocytes and processes of growth in the thymo lymphatic system (see *Otani* 1957, *Imamura* 1959, *Ernstrom & Larsson* 1961) The growth of the thymus in mice is very intensive during the first two weeks of life, when a great number of "large free round" cells appear in the cortex (*Axelrad & van der Gaag* 1962) After the age of 2 or 3 weeks these cells are replaced by smaller lymphoid cells The "large free round' cells seem to correspond to the large pyroninophilic cells, rich in mitochondria, observed in the thymus of guinea pigs by *Ernstrom & Larsson* (1963) Experimental work by *Miller* (1961) indicates that the thymus plays an important rôle perinatally for the development of a lymphatic tissue with normal immunological reactivity The present findings of lymphocytes rich in mitochondria circulating in the blood of mice during the first postnatal week might correspond to the large thymic cells observed by *Axelrad & van der Gaag*, and might also represent the progenitors of the immunologically competent cells in the body, migrating from the thymus

Although the oxygen-exposed animals showed a retarded growth and other developmental abnormalities, the number of mitochondria in their blood lymphocytes did not differ significantly from the control values of normal animals This probably indicates that a high concentration of oxygen does not influence the number of mitochondria in the white blood cells If, however, the mitochondrial contents are correlated to general development instead of age, the oxygen-exposure decreased the number of mitochondria in the blood lymphocytes

SUMMARY

The postnatal change of the mitochondrial contents of the blood lymphocytes was studied in normal mice and in animals exposed to concentrated oxygen from birth During the first week of life, coinciding with the intensive postnatal growth of the thymus, the blood lymph-

ocytes contained a relatively large number of mitochondria From the second week on the contents decreased rapidly The oxygen exposed animals showed a retarded growth postnatally, but did not differ from the normal control animals with regard to the contents of mitochondria in their blood lymphocytes

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POTENTIATION OF SENSITIVITY TO RENIN IN NORMAL RATS BY CROSS CIRCULATION WITH NEPHRECTOMIZED

By

JENS BING and FRANK MAGILL

Received 13 xi 1963

The phenomenon of increased and prolonged pressor response to injections of renin in the nephrectomized animal is a well established entity, first described over 60 years ago by *Tigerstedt & Bergman* (1898). The mechanism(s) whereby this occurs, however, remains obscure. Two recent studies by different authors, who have attempted to determine whether or not this renoprival effect could be related to some humoral factor, have produced contradictory findings. *Gross et al* (1962) in a large series of cross circulations between normal and nephrectomized rats, found that the nephrectomized animals showed a subsequent decrease in their pressor sensitivity to renin, the normal animal remaining

to subsequent injections of renin. The parameters of renin sensitivity in these two groups were different. The *Gross* group using the integral of the pressor curve for 30 minutes following the injection of the renin, while the *Blaquier* group used the elevation of blood pressure in mm of Hg and does not mention the duration of response.

The present experiments were undertaken with the aim of reinvestigating the possibility that the increased renin sensitivity of the renoprival rat is related to some humoral principle which might be transferred by cross circulation to a normal animal.

MATERIAL AND METHODS

52 female albino rats weighing 200 grams (±10 grams), were used. These were divided as follows

Supported by grants from "P. A. Brandt's Foundation", "King Christian X's Foundation" and "Nordisk Insulinfoundation" (to Jens Bing) and "The Squibb Fellowship of the Endocrine Society" (held by Frank Magill).

We express our thanks to "Ciba", "Ferrosan" and "Leo" for having supplied us with angiotensin, renin, heparin and penicillin.

a normal animals cross circulated with other normals (Group 1) 8 pairs
b nephrectomized animals cross circulated with other nephrectomized (Group 2) 2 pairs
c nephrectomized animals (Group 3) cross circulated with normals (Group 4) 16 pairs

After i m injection of 45 000 Int Units of Dipenicillin Leo, bilateral nephrectomy was carried out under ether anaesthesia 24 hours prior to cross circulation In the interval the animals were allowed free access to food and water

Animals were prepared for cross circulation and testing of renin sensitivity in the following manner Sodium Amytal was given intraperitoneally (10 mgm per 100 grams body weight) The left carotid artery and jugular vein were exposed and cannulated using polyethylene tubing (size P E 90) *filled with a solution of heparin* (1000 I U./ml) and occluded at their distal ends by screw clamps A tracheal cannula was inserted at the same time The incision was then closed using metal skin clips to prevent bleeding After an interval of 10 to 15 minutes to allow for clotting of the wound 200 I U *of heparin were administered through the venous* cannula

The animals were then connected to the cross circulation apparatus described by *Brodish & Long* (1956) 1 with the modification that instead of the femoral vessels we used the *jugular and carotid vessels The major advantage of this technique* is that by using a graduated drip chamber between arterial output and venous input and air pressure to force blood from the chamber the input rate can be made to correspond exactly to the outflow and constant blood volume in both animals is *maintained throughout the cross circulation procedure Use of the neck vessels* allows for a very extensive exchange in a short periode of time In the present series of experiments 30 to 40 ml of blood were exchanged in 15 to 20 minutes giving close to 95 per cent mixing between the two animals This was calculated from the *result of a series of cross circulations by Brodish & Long (1956) using radioactive* Fe^{59} labelled red blood cells in one member of a pair and then plotting their rate of appearance in the other member against the volume of blood cross circulated

After cross circulation, the arterial cannula was disconnected from the apparatus *and reconnected to a mercury manometer and kymograph The venous cannula was* used for injections Each animal was tested with 5 ng synthetic val—5—angiotensin II amid (hypertensin Ciba) followed by 1 unit of our hog renin preparation *which* is the amount which in our normal rats gives an increase in blod pressure of about *12 mm this dose being* about *1/40 of a* Goldblatt Dog Unit

On the completion of the testing arterial blood was collected and assayed for its *ratio of angiotensin yield* The method for this determination was as follows

1 ml of plasma was added to 2 ml of a horse angiotensinogen preparation *After mixing they were preheated to 37° C and further 1 ml of a similarly* preheated hog renin preparation diluted with a phosphate buffer (pH 7 5) was added The mixture was incubated for 10 min at 37° C boiled centrifuged and the supernatant tested for angiotensin the yield being compared with a control preparation in which 1 ml *of an 9 9 per cent NaCl solution was substituted for the plasma* The ratio of angiotensin yield is thus

$$\frac{\text{angiotensin yield in test with plasma}}{\text{angiotensin yield in control with 0 9 per cent NaCl}}$$

Before testing angiotensinase was destroyed both in the angiotensinogen preparation and in the plasma samples by subjecting them to a pH of 3 5 at 25° C for 20 *min The renin preparation was diluted* so that 1 ml contained about one half Goldblatt dog Unit (as compared with the commercially available renin preparation from Nutritional Biochemical Corporation Cleveland Ohio) In this dilution the renin preparation was without angiotensinase effect when tested with synthetic *angiotensin* (Hypertensin Ciba) for 10 minutes With this standard technique angiotensinogen was in excess This was shown by using two to four times the angiotensinogen concentration in the mixture which did not influence the yield of angio

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1 We wish to thank Dr *Hans Bohr* who first used the technique at this institute and introduced the present authors to it

tensin and by using higher or lower renin concentration, which resulted in similarly varying angiotensin formation

[illegible] 3 mg/kg body

RESULTS

Our findings in a total of 36 animals are given in Fig 1 to 4 (Of the 52 animals with which we started, 16 could not be tested because of difficulties with the apparatus in the early phase of this work)

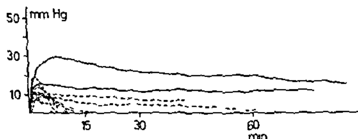


Fig 1

Response to renin in 9 normal rats cross circulated (95 per cent mixing) with other normal (broken lines) and in two nephrectomized animals cross circulated (95 per cent mixing) with other nephrectomized rats (unbroken lines) Abscissa in minutes. Ordinate increase in blod pressure in mm Hg

A Response to Renin

I Cross circulation between two normal rats Fig 1 gives the result of *testing the sensitivity to renin in 9 normal rats after cross circulation with* other normal rats, the cross circulation in all cases giving an estimated per cent of mixing of 95 Nearly all animals *respond* with *the* normal pattern of response a rapid blood pressure *rise*, usually 5 to 15 mm of Hg, immediately following the *injection of renin*, *a* short-lived plateau for 1 to 2 minutes, and then a gradual *fall to the* initial base line within 15 minutes One animal had *a higher maximal blood* pressure elevation (18 mm), but a normal *duration of response* (10 minutes) Two others had a normal height (7 and 10 *mm*) *but an* increased duration (> 40 and 60 min), *the cause of* which is unknown

II Cross circulation between two nephrectomized rats The result of renin tests on two rats of this group (95 *per cent mixing*) is given in Fig 1 Both animals reacted with a *potentiated* pattern of response, one of them giving a maximal blood *pressure rise* in *the* normal level (13 mm) but having a prolonged pressor response with a plateau close to this maximal value, the *other having* both an increased maximum value (20 mm) and a prolonged curve at a *high level*

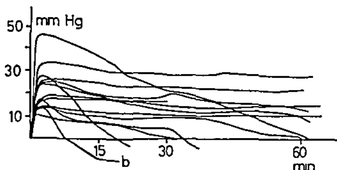


Fig 2
Response to renin in *13 nephrectomized rats* after cross circulation with normal rats The curve marked b is from a rat with bleeding from the wound Abscissa and ordinate as in Fig 1

III Cross circulation between a normal and a nephrectomized rat The sensitivity to renin was studied in *13 nephrectomized rats after cross circulation with normal rats,* the estimated per cent of mixing being in two cases 55, in two cases 80 to 85, and in 9 cases 95 Fig 2 shows that nearly all of them reacted with the potentiated pattern of response with both increased height and duration of response seen in group II, only two responding with a duration of less than 30 minutes, one of these having a maximal height of 27 mm The other animal (marked b) had a quite normal curve, which is believed to be due to bleeding, which also is thought to be the cause of the continued fall below the base line observed in this animal

A corresponding determination of the sensitivity to renin was performed in *12 normal rats after cross circulation with nephrectomized rats,* the estimated per cent of mixing being 80 to 85 in three, and 95 in 9 cases Fig 3 shows that while the three animals with 80 to 85 per cent mixing reacted to renin in the same way as the normal rats in group I (Fig 1), the nine animals with a 95 per cent mixing all reacted with the potentiated pattern found in nephrectomized rats (Fig 1 and 2)

B Response to angiotensin

While the differences in renin sensitivity between normal and nephrectomized rats was pronounced, no similar differences were found in the response to angiotensin In accordance with this no significant differences were found between the angiotensin sensitivity of normal rats cross circulated with other normal rats, and the sensitivity of those which had been cross circulated with nephrectomized rats

C Angiotensin Yield

In previous studies (*Bing* 1964) it was found that the normal value for this measurement is usually from 1 to 1 5 units, while the values seen in animals that have been nephrectomized for 24 hours are almost

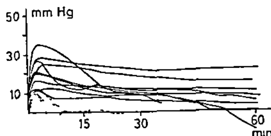


Fig 3

Response to renin in *12 normal rats* after cross circulation with nephrectomized animals The cases with 95 per cent mixing are marked with unbroken lines, the broken lines indicating 3 experiments with from 80 to 85 per cent mixing Abscissa and ordinate as in Fig 1

always in the range of 2 to 4 units and occasionally as high as 7 In the present studies (Fig 4A) we found that the normal control group (homologous cross circulation of normal with normal) had values of 1 5 units or less in 5 of 6 cases and in the other case the value was 2 units Similar determination of the yield in two cases of homologous cross circulation of nephrectomized with nephrectomized gave values of 3 in both cases The normal animals that had been cross circulated with nephrectomized animals had elevated values in 9 of 11 cases (Fig 4B) One of the animals of the latter group whose value was in the normal range was one of those in which cross circulation had been limited to 80 per cent mixing and the transfer of increased renin sensitivity did not occur The elevated angiotensin yields in groups III and IV parallel rather closely the increased sensitivity to renin

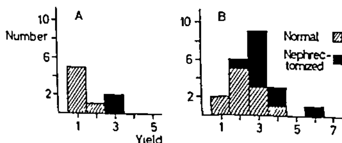


Fig 4

The yield of angiotensin formation is given by the ratio between the values obtained by the following two incubations

$$\frac{\text{angiotensinogen + plasma + renin}}{\text{angiotensinogen + saline + renin}}$$

the amount [illegible] en after homo
l g us (A) a [illegible] and nephrecto
mized rats [illegible] increased after

D Change of Sensitivity to Renin after Intravenous Injection of 2 ml of Plasma from Nephrectomized Rats and Guinea Pigs

While the sensitivity to renin was unaffected by intravenous injection of 2 ml of plasma from normal rats (5 experiments), similar injections of plasma from nephrectomized rats or guinea pigs resulted in a significant potentiation in all of 5 experiments In both groups the sensitivity to angiotensin was either unaffected, decreased, or changed to a depressor effect after the plasma injections

DISCUSSION

The present studies have shown, that both the potentiation of sensitivity to renin in nephrectomized rats and the increased yield of angiotensin obtained by incubation of their plasma with renin can be transferred to normal rats by means of either cross circulation or plasma transfusion These results contrast with the conclusion of *Gross et al* (1962), but confirm the conclusion of *Blaquier et al* (1962), showing that the potentiation phenomenon of the nephrectomized rats is due to a humoral factor

SUMMARY

The phenomenon of potentiated renin sensitivity in the nephrectomized animal was studied by cross circulating normal with nephrectomized rats It was found that when cross circulation was carried out to the extent of 95 per cent mixing between the two animals, the normal animal would subsequently respond to injections of renin as though it had been nephrectomized, and that incubation of its plasma with renin gave an increased angiotensin yield Similarly a potentiation of the response to renin was found after intravenous injection of about 2 ml of plasma from neprectomized rats or guinea pigs into normal rats The potentiation phenomenon must thus be related to some humoral principle(s)

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STUDIES ON THE POSSIBLE ANTI-NEOPLASTIC EFFECT OF THALIDOMIDE

By

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Received 31 x 63

The discovery of the pronounced teratogenic properties of thalidomide has naturally led to reflections on the possible power of the drug to influence the growth of tumour cells. However, as far as we know, no experimental studies on the chemotherapeutic activity of thalidomide in the treatment of cancer have been published, although some authors have suggested that such studies should be performed. On the other hand, a few reports on the treatment of hopelessly sick cancer patients are on record. In these cases, the treatment was without any effect on the tumour growth.

The first report on the use of thalidomide in the chemotherapy of cancer seems to be that published by *Woodyatt* in a Letter to the Editor of the Lancet (1962), in which he briefly stated that he had treated a woman with a X-ray resistant pelvic tumour with thalidomide (Distaval) 400 mg daily for eight days. The tumours increased in size during the treatment. This brief communication appeared after (*Rogerson* 1962) had published a paper on thalidomide abnormalities, which ended with the following sentence: "One wonders whether this drug, which seems to have such remarkable inhibitory powers on growing tissues in certain cases, is being investigated for possible anti carcinogenic properties".

The mode of action of thalidomide of foetal development is unknown, but the interference must occur at a very early stage of the embryogenesis, presumably in particular during the critical phase of limb development (in man in the 4th–6th weeks of pregnancy). Thalidomide may give rise to various foetal deformities, most frequently of the limbs,

Aided by grants from the *Anders Hasselbalch Anti Leukaemia Foundation*, *Carl Schepler and Wife's Bequest*, the *Irma Foundation* and the *Danish Anti-Cancer League*.

Thalidomide is a phthalidomido glutarimide (N phthalylglutamic acid imide) and is also known by the following trade names: Contergan, Neosedyn, Valgis, Valgrain, Asmaval, Distaval, Softenon, Quitemide, Calmorex, Kevadan, Isomin, Telargan, Tensival, Lullamin and Sedalis.

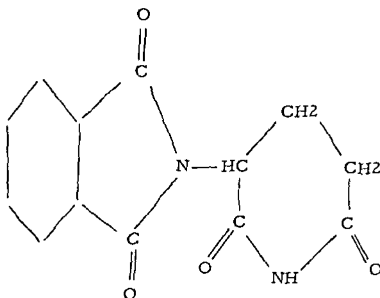
usually resulting in amelia or phocomelia It must be assumed that highly sensitive, undifferentiated tissues which are of importance for the normal development of the limbs occur at that time

*In view of our complete lack not only of effective agents in the chemo*therapy of cancer, but also of a rational basis in the search for such agents, the observation that thalidomide has pronounced teratogenic powers is sufficient to justify an investigation into its possible anti-neoplastic properties As a rule, tumours contain a large number of *undifferentiated cells* which are highly sensitive to any harmful effect

One report seems to suggest that thalidomide *may* exert a direct influence on cells—on a cellular level *Roath et al* (1962, 1963) observed that thalidomide inhibits lymphocytes in tissue culture The transformation of small lymphocytes into large cell forms which occurred in the cultures studied by these authors was partially arrested when thalidomide was added to the cultures

It was therefore only natural that we studied the anti-neoplastic effect of thalidomide on some of the transplantable mouse tumours which we have available for such purposes

Thalidomide, which is a derivative of glutamic acid, was synthetised by *Kunz, Keller & Muckter* in 1956 In the pure state, it is a tasteless, crystalline substance, which is only slightly soluble in water, methanol and ethanol, but freely so in ether an benzene Its formula is as follows



As early as 1956, *Kunz et al* observed that thalidomide was relatively non-toxic and wrote that mice "virtually tolerated the maximum dose 5 g/kg" They also reported that the agent produced sedation without preceding excitation The new drug had a pronounced hypnotic effect, but did not induce anaesthesia Even after a large hypnotic dose the

animals could be aroused They did not present signs of incoordination Thalidomide does not suppress convulsions induced by strychnine or electroshock It is not analgesic and does not affect the normal body temperature, but is yet capable of reducing a temperature rise produced by injection of inactivated E coli into rabbits Blood pressure, pulse rate and respiration are not effected All these early observations have later been confirmed In his series of 100 thalidomide treated patients, *Schober* (1959) found that haematopoiesis remained unaffected

Lüers (1962) studied the mutagenicity of thalidomide The experiments, which were performed on drosophila (standard Müller-5 technique), failed to show any signs of mutagenic action on sperms or early spermatogenesis

Hughes et al (1962) found no changes in the karyotype in two abnormal foetuses borne after the intake of thalidomide (Tensival)

It was natural enough that this hypnotic agent, which had so slight side effects which did not produce excitation before sleep, and which, in addition, could be given without any risk of suicide, soon became widely used in many countries The drug was given as a hypnotic in doses of 100–200 mg in the evening or as a sedative in single doses of 25 mg 3–4 times daily Its low toxicity is strikingly illustrated by the fact that all attempts at committing suicide with thalidomide as the only drug have "failed' (*de Souza* 1959, *Burley* 1960)

In 1960–1961, a number of authors suggested almost simultaneously that thalidomide might give rise to toxic neuropathy (*Bartholomew* 1961, *Chevens* 1961, *Coster* 1963 *Florence* 1960, *Fullerton & Kremer* 1961, *Heathfield* 1961, *Howe* 1961, *Kuenssberg* 1961, *Scheid et al* 1961, *Shafar* 1961) Owing to the presumed neurotoxicity, A/S GEA which is the *distributor in Denmark, withdrew the drug from the market* Shortly afterwards, the first reports on the teratogenic properties of thalidomide appeared This possibility was first considered and discussed by *Lenz* [illegible]
togenicit [illegible]
1962 *H* [illegible]
section of this paper, it was briefly mentioned that the most frequent deformity is limb malformations if the drug is taken during the early weeks of pregnancy in man The teratogenic effect of thalidomide has been demonstrated experimentally in various animal species Sprague Dawley rats (*King & Kendrick* 1962), mice and rats (*Felisati* 1961), rabbits (*Somers* 1960), and hens (*Kemper* 1962)

On oral administration of thalidomide about 40 per cent [illegible]

with the metabolism of fat and carbohydrate In the metabolism of amino acid and protein it plays a major role as an—NH_2 donor *Leck & Millar* 1962 *Faigle et al* 1962 *Beckmann* 1962 and *Beckmann & Kampf* 1962 suggested that thalidomide may interfere with the metabolism of riboflavin since riboflavin deficiency is capable of producing malformations in rats In this connexion it is of interest that several authors have observed glossitis and other oral lesions as well as fissures at the angles of the mouth during treatment of patients with thalidomide (*Mancini* 1962 *Leck & Millar* 1962) *Robertson* (1962) wrote that since using vitamin B complex prophylactically gross mouth lesions have been largerly *controlleed* *Millan* (1962) enumerated a large number of substances which are capable of producing foetal malformations when given in over or under dosage This enumeration included deficiency of pantothenic acid or of folic acid It is known that the folic acid *antagonist aminopterin is teratogenic also in non lethal doses Other* anti neoplastic chemotherapeutic agents such as Myleran and Chlorambucil are also known to be teratogenic

MATERIAL AND METHODS

In our investigations on the possible anti neoplastic effect of thalido mide we used three of our own transplantable mouse tumours

1 *The NJA tumour* is a transplantable leukaemia which arose spontaneously in 1960 in the C_3H/a strain in which leukaemias are otherwise rare It is a generalised stem cell leukaemia with very pronounced blood changes (see *A Olsen* 1963) The leukaemia is transplanted either by subcutaneous inoculation of a suspension of tumour cells (approx 10^6 cells per μ 1) into an axilla or by inoculation of heart blood After both f*orms of transplantation the animals die very regularly on the* 9th or 10th day (see the curves for the control animals in the figures) Takes and progressive growth are obtained in 100 per cent of the inoculated C_3H/a mice Spontaneous regression does not occur We have not succeeded in establishing the *tumour as an ascitic tumour*

As a consequence of the uniform tumour growth and the uniform survival time of the animals after the inoculation this tumour is very suitable for chemotherapeutic experiments

2 *The PBH tumour* is an adenocarcinoma which arose in *1951 in the breast of* a C_3H/a mouse in the Laboratories of the Radium Centre for Jutland The tumour has since been transplanted routinely by subcutaneous injection of tumour pulp into an axilla The tumour has now been carried through 290 generations At first the tumour was a highly differentiated adenocarcinoma but in 1952 it changed in character almost into an anaplastic solid carcinoma (*A Nielsen* 1962)

3 *The GH tumour* displayed certain irregularities For example growth failed to occur in some animals both in the experimental and the control group Accordingly these experiments were excluded

In all cases the experimental animals were C_3H/a mice which have been inbred here in Aarhus for several years

EXPERIMENTAL PROCEDURE

The C_3H/a mice used in the experiments were about 2 months old The animals were divided into groups of 6 or 10 as far as possible consisting of an equal number of males and females and of animals of the same weight Both the NJA and the PBH tumour were transplanted by inoculation of a suspension of 10^6 tumour cells in sterile physiological saline into one axilla The injection fluids were given daily partly sub cutaneously *on the right side* and partly intraperitoneally

As thalidomide[1] is only slightly soluble in water at 37° C (approx

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1 Thalidomide was *kindly* supplied by A/S CFA Copenhagen

15 mg per 100 ml), and as the substance is rapidly decomposed in alkaline environment, it was necessary to prepare the injection fluids as suspensions in order to administer the amounts desired The suspension medium was physiological saline to which 1 per cent Tween 80 was added All the thalidomide suspensions were prepared in such concentrations that the injected amounts were invariably 0 3 ml In all the experiments reported below, thalidomide was given in the following doses

1) 11 2 mg kg 3) 560 0 mg kg
2) 112 0 mg kg 4) 1120 0 mg kg

(In comparison it may be stated that, in man, the hypnotic dose is 100-200 mg *i e* 14 3 28 6 mg kg)

THE INDIVIDUAL EXPERIMENTAL RESULTS

In all the experiments, the injections of thalidomide were commenced on the day following that of the tumour transplantation In order to avoid that the illustrative diagrams become too long, the time of transplantation is indicated on the abscissa as the starting point, while the days until the first deaths occur have been compressed The dead animals were subjected to autopsy, and the spleen, liver, kidneys, lungs and tumour were examined histologically

Experiment 1 comprised five groups of six C3H a mice transplanted with the PBH carcinoma The thalidomide suspension was injected sub cutaneously (Fig 1) As is seen from the curves, all the thalidomide

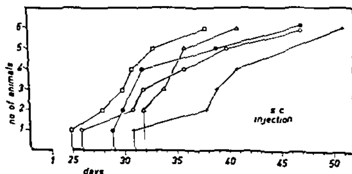


Fig 1

Experiment 1 comprising fiv groups of six C3H/mice transplanted with the PBH tumour and treated with daily subcutaneous injections of thalidomide which were commenced on the day following that of transplantation Thalidomide was given in daily doses as follows

□ 28 000 mg
△ 2 800 mg
● 0.280 mg
○——○ 0 028 mg
×——× Solvent

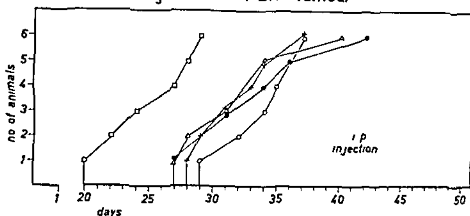


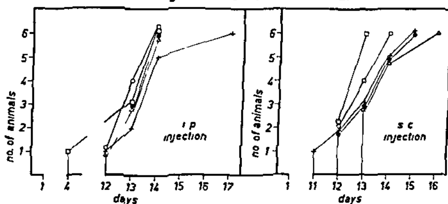
Fig 2

Experiment 2 Animals tumour and doses as in experiment 1
Thalidomide was here given intraperitoneally

treated animals died a little before the controls, apparently, the larger the dose of thalidomide, the shorter is the period of survival

Experiment 2 was performed as 1, but here thalidomide was given intraperitoneally It is seen from the curves (Fig 2) that the experimental and the control animals died almost simultaneously However, the animals which had received the largest dose of thalidomide (28 mg) died earlier than all the others

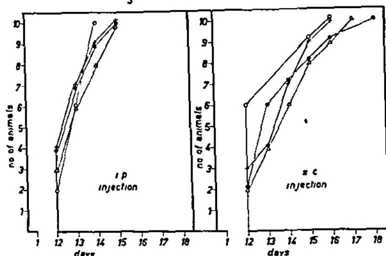
Experiment 3 was performed with NJA tumour transplanted into



Figs 3 a and 3 b

C_3H/a mice transplanted subcutaneously with the NJA leukaemia The thalidomide treatment was commenced on the day after the transplantation and continued with daily injections given subcutaneously in 3 a and intraperitoneally in 3 b (In one case, the abdominal organs were presumably injured by the intraperitoneal injections since the animal died three days after the first thalidomide injection) Thalidomide was given in daily doses as follows





Figs 4 a and 4 b

These experiments comprised five groups of 10 C_3H/a mice transplanted with the NJA tumour and treated with daily injections of thalidomide given intraperitoneally in 4 a and subcutaneously in 4 b Thalidomide was given in daily doses as follows

△———△	2 800 mg	○———○	0 028 mg
●———●	0 280 mg	×———×	Solvent

C3Ha mice, but otherwise the procedure was as mentioned above As before, these experiments did not reveal any significant effect of thalidomide on the survival times of the transplanted animals, nor did histological examination show any differences with regard to the extent of the leukaemic infiltrations in the organs (Fig 3)

Experiment 4 was performed as 3 The various groups did not reveal any significant differences (see Fig 4)

Experiments 5 and 6 were performed in the same way as 3 and 4, and the results were also the same

DISCUSSION

Mice are relatively insensitive to thalidomide Even doses which are many times larger than those which are definitely hypnotic in man do not produce any visible sedative effect in the animals (dose determined per kg body weight) Owing to our lack of knowledge of this low sensitivity our first tumour-therapy experiments were performed with very small doses In our subsequent experiments described in this paper, the dose of thalidomide used in the groups of animals which received the largest amounts was 1000 times as high as the hypnotic dose per kg body weight in man These large doses induced sleep for some hours in the animals, which could, however, always easily be aroused No other

effect were observed in the animals. Even when under treatment with large doses, the animals took nourishment normally, and the weight curves for the experimental animals closely followed those of the controls.

In none of the experiments did thalidomide reveal any anti-neoplastic effect. It may be objected that thalidomide was given subcutaneously and intraperitoneally, and not by mouth. However, it must be supposed that a possible anti-carcinogenic effect would nevertheless have manifested itself, since the very high dosage produced sleep, from which the animals could easily be aroused, *i.e.* an effect similar to that known in man after oral administration of the drug. In addition, the conclusions which may be drawn *from our experiments are limited by the fact that* we employed only one mouse strain and two tumours which had arisen in the same strain, *viz.* an adenocarcinoma and a leukaemia.

SUMMARY

Thalidomide was studied experimentally with a view to a possible anti-carcinogenic effect on a carcinoma and a leukaemia in mice. Even very large doses of thalidomide did not show any inhibitory effect on the tumour growth.

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HISTOLOGICAL DEPARTMENT AND INSTITUTE FOR MEDICAL GENETICS
UNIVERSITY OF UPPSALA, SWEDEN

ISOZYME PATTERNS IN CHICKEN PANCREATIC TISSUE[1]

By

BO HELLMAN and L. BECKMAN

Received 14. vi. 63

The combination of improved methods for electrophoretic separation, such as starch gel electrophoresis (*Smithies* 1955) and histochemical *staining methods makes it possible to study different molecular forms* of enzymes (*Hunter & Markert* 1957). The concept of an isozyme has been defined as one of the different molecular forms of an enzyme (*Markert & Moller* 1959), and the electrophoretic strip stained for enzymes is called a zymogram. It is now well established that different proteins with similar enzyme activities may exist in the same organism and even within the same tissue (*Markert & Hunter* 1959).

The present investigation deals with the distribution and isozyme variations of both napthylesterases and enzymes hydrolyzing the chromogenic substrate L-leucyl-β-napthylamide within the pancreas. The zymograms obtained from the pancreatic tissue were compared with those of serum and liver extracts. Chickens were selected for these studies, since the topographical distribution of the islet tissue within the pancreas of this species allows analyses of pancreatic portions, which either contain as much as 15–20 per cent A cells or practically lack such endocrine cells. While the dark islets (= A cells islets) are confined to the 3rd and splenic lobes, the light islets (= B cells islets) are scattered throughout the gland (*Mikami & Ono* 1962).

MATERIAL AND METHODS

Animals. Male chickens (white Leghorn) weighing 600–700 g were used. The animals were allowed free access to food and water and killed by decapitation.

Histochemistry. Small blocks of pancreatic tissue from six chickens were rapidly excised from the splenic and ventral lobes. The tissue samples were plunged into isopentane chilled to —70° C in a bath of dry ice and alcohol and sectioned in a cryostat at —20° C. The tests for esterases were made according to *Gomori* (1952) by incubation in a medium with α-naphthylacetate as substrate and Fast Blue B salt as diazonium coupler. The enzymes hydrolysing L-leucyl-β-naphthylamide (= the naphthylamide reaction) were studied by the method of *Nachlas et al.* (1957).

Zymograms. Homogenates were prepared from the liver (*L*) as well as the ventral (VL) and splenic (SL) lobes from 36 chickens. Material from 3–6 chickens was pooled and homogenized with distilled water. The homogenates were frozen-thawed

1 Supported by the *Swedish Medical Research Council.*

10 times and then centrifuged The supernatant solution and serum samples (S)

RESULTS

A brown staining of esterases was present throughout the exocrine and endocrine pancreatic parenchyma The dark islets (= A cell islets) contrasted against this background in showing a black reaction around the capillaries (Fig 1) Whether the latter type of reaction also included the capillary poles of the islet cells could not be definitely decided With the naphthylamide reaction no appreciable staining was recorded in the sections, except for a moderate violet reaction in the excretory duct epithelium and a faint one in the dark islets

Starch gel electrophoresis and subsequent staining with Amidoblack 10B revealed that the protein patterns were entirely different in serum, liver, and pancreas (Fig 2) The serum proteins resembled what has been observed in other vertebrates In the liver homogenate there were only some fast moving components, not observed in the serum but also present in the pancreas extract The pancreas homogenate had a large number of protein components, a great similarity being noted between the ventral and splenic lobes as regards the distribution and concentration of the different proteins (Fig 3)

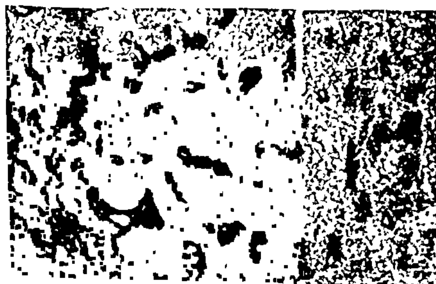


Fig 1

Esterase activity in a dark islet (center) from a pancreatic section counterstained with haematoxylin There is a marked reaction along the capillaries

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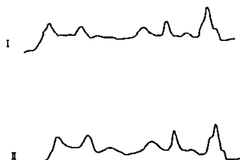


Fig 3
Scanning patterns of electrophoretic strips stained for proteins I = splenic pan creatic lobe II = ventral pancreatic lobe There is a marked similarity of the protein patterns for the two pancreatic portions

Application of the naphthylamide reaction revealed two different components of varying strength in the zymograms from the liver and the pancreas Only the slower of these components was present in serum In addition there was an extra slow moving fraction in serum (see Fig 2) The relative concentrations of the two components in the pancreatic zymograms were different in the two lobes It was repeatedly

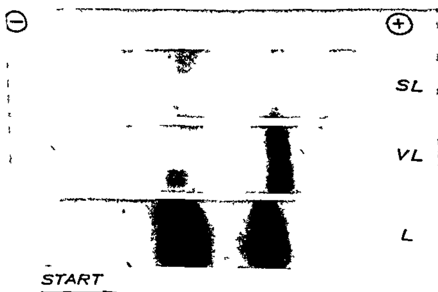


Fig 4
Zymograms showing zones with a positive naphthylamide reaction SL = splenic pancreatic lobe VL = ventral pancreatic lobe and L = liver Note the difference in the relative concentrations of the two fractions in the two pancreatic portions

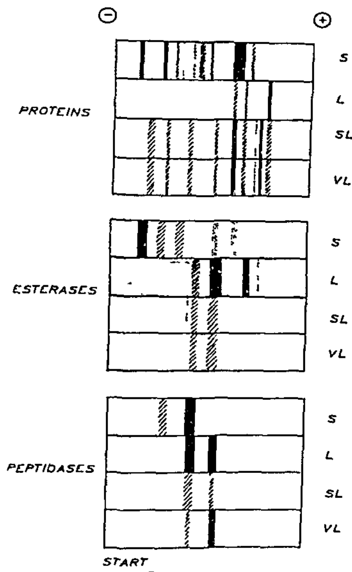


Fig 2

Amido black protein patterns and zymograms showing esterases and enzymes splitting leucyl β naphthylamide S = serum L = liver SL = splenic pancreatic lobe VL = ventral pancreatic lobe

Distinct and organ specific isozyme patterns were noted for the esterases Five components were observed in serum The slow moving zone with high activity, usually found in vertebrate sera, is apparently lacking in liver and pancreas A rather intense enzyme staining was found all over the liver zymogram from the start up to the albumin region This might obscure eventual small distinct fractions in the slow moving region In both the splenic and ventral lobes two distinct esterase zones were found, no obvious differences being noted between the two pancreatic parts

the chicken pancreas has been demonstrated in the duck (*Arvy* 1960, *Hellerstrom* 1963) It seems reasonable to assume that the marked esterase reaction noted along the capillaries of the dark islets comprises the capillary pole of the argyrophil A_1 cells (*cf* *Hellerstrom* 1963) Recent electron microscope studies in the duck (*Bjorkman & Hellman* 1963) offer no support for *Arvay's* (1960) suggestion that the latter reaction is accounted for by a rich cholinergic innervation It emerged from the present studies that the esterases around the capillaries of the dark islets probably represent the same molecular forms of enzymes, which is responsible for the diffuse histochemical staining of the remaining [illegible] o obvious differences [illegible] of the splenic lobe, [illegible] ll islets

It is now well established that the chromogenic substrate L-leucyl β naphthylamide, originally used for demonstration of leucine amino peptidase (*Burstone & Folk* 1956, *Nachlas et al* 1957), is hydrolysed to different extents by several enzymes (*Sylven & Bois* 1962) The potentialities of the naphthylamide reaction for rough screening of available proteolytic activities is, however, apparent and it was therefore used in the present study The same enzyme peaks were observed in the zymograms from the liver and pancreas The observation that the slower of these zones was the most intense in the splenic lobe but the weaker in the ventral lobe may indicate that the enzyme splitting of L-leucyl-β-naphthylamide in the dark islets is associated with this particular peak In view of the rather low activities in the chicken A cells of these enzymes great caution is, however, warranted in drawing any definite conclusions Species differences apparently exist for the enzyme distribution within the bird pancreas, since *Hellerstrom* (1963) found a pronounced naphthylamide reaction as characteristic for the cell content of the dark islets of the duck

SUMMARY

The isozyme patterns of chicken pancreas were compared with the corresponding zymograms obtained from serum and liver Organ specific electrophoretic differences were noted both for proteins stained with Amidoblack and for esterases No differences were observed between esterase zymograms from the splenic pancreatic lobe, rich in A cells and the ventral lobe The marked esterase reaction along the capillaries of the A cell islets is therefore probably due to the same isozymes as those responsible for the diffuse histochemical staining in the remaining pancreas

The application of the naphthylamide reaction revealed two electrophoretic zones in the pancreas A quantitative difference between the pancreatic lobes indicates that the enzyme activity histochemically found in islet A cells might be associated with the slow moving zone

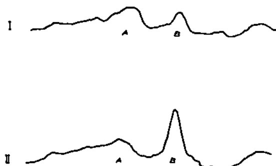


Fig 5

Scanning patterns of zymograms showing pancreatic naphthylamide reaction I = splenic pancreatic lobe, II = ventral pancreatic lobe Note the difference between the relative proportions of peaks A and B in the two lobes The absolute concentrations of the two homogenates are not comparable

found that the slow moving component in the extracts from the splenic lobe occurred in a relatively higher concentration than that of the ventral lobe (Figs 4 and 5)

DISCUSSION

The protein stain with Amidoblack 10B revealed entirely different patterns in serum, liver, and pancreas Organ specific zymogram differences were noted for the esterases splitting α-napthylacetate The slow and strongly staining zone of serum, which most likely is homologous with the slow cholinesterase peak found in human serum (*Harris et al* 1962), is lacking in pancreas and apparently also in liver In a comparative study of esterases present in plasma of 27 vertebrates *Augustinsson* (1961) concluded that each species has its own esterase pattern While arylesterase was the predominant esterase in mammalian plasma, it was absent in lower vertebrate plasma including birds The electrophoretic behaviour of esterases from chicken serum and liver was studied by *Paul & Fottrell* (1961) While these authors reported 4 esterase peaks in the starch zymogram for the serum samples, 5 peaks were identified in the present system The weak and most fast moving peak in our liver extracts does not seem to have been reported previously

A comparison of the present esterase data and some previous observations regarding lactic dehydrogenase (LDH) raises a point of interest *Vesell* (1961) and *Wroblewski & Gregory* (1961) reported 5 zones of LDH activity on the starch gel zymograms The same 5 activity peaks

tissues studied with an organ specific

in these peaks Liver and skeletal

peak 1 (the slowest one) leucocytes

and serum in peak 4 and pancreas in peaks 3 and 5 Thus in the case of LDH the isozyme differences are quantitative in nature, while those found for the esterases are strictly qualitative

A similar distribution of esterase activity as noted in sections of

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SEROLOGICAL STUDIES ON HAEMOPHILUS INFLUENZAE AND RELATED SPECIES

4. *Examination of the Type Specific Antigens of H. influenzae by Means of the Gel Precipitation Method*

By

TOM OMLAND

Received 14.ii.63

The adaptation of the gel precipitation method to the study of type specific antigens in Haemophilus influenzae has been described (*Omland* 1963c). In this work certain type reference systems were used, some of which contained, however, considerable amounts of non type-specific elements, rendering interpretation difficult. It was thus obvious that efforts had to be made to improve the reference antigens and antisera before they could be used as instruments in the subsequent work.

Standardization of Reference Antigens and Antisera

Antigens. A series of experiments was performed, in which fractions of the antigens were separated by precipitation with ethanol. The same type reference strains were used as those employed in earlier parts of this study (Omland 1963b, c), namely a 51, b 51, c 51, d 51, e 51, and f 51. The modified *MacPherson's* procedure (1948) described earlier (*Omland* 1963c) was followed up to the first precipitation with ethanol. Instead of a single precipitation with 5 volumes, fractionated precipitations with 1, 2, 3 and 5 volumes were carried out. The fractions were otherwise treated in the same way as described earlier. The *antigen* fractions thus obtained were set up in gel precipitation experiments against homologous type antisera. Antigens prepared by one precipitation with 5 volumes of ethanol were used as controls. These control antigens were identical to those employed in a previous examination (*Omland* 1963c, Table 1 and Fig. 1). The present examinations yielded clear-cut results in the types a, b, c, and e. Concerning ethanol precipitation characteristics the type specific antigens fell *in two groups*, the first consisting of types a, b, and c, represented by the B system (Fig. 1), the second consisting of type e (E-system, Fig. 1). In the first group the type specific antigen was precipitated already by 1 volume of ethanol. In type e, however, 3 volumes were needed for the precipitation of the type specific antigen. These experiments showed, on the other hand, that non type-specific *antigens* generally required more

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At the time of these examinations satisfactory sera for types d and f were not available As soon as such sera were obtained, similar experiments were carried out in these types, which were shown to possess ethanol precipitation characteristics analogous to the types a, b, and c (first group) Concerning certain physico-chemical properties the e-antigen used thus appears to occupy a unique position compared to the other type antigens

The prepartion of type reference antigens was subsequently carried out according to *MacPherson*s procedure (1948), modified in accordance with these results A review of this work is presented in Table 1

Considering the amount of media and work involved, the yield of type reference antigens was very low, and consequently it was decided not to proceed with further refinement of the prepared substances Even though no attempt was made at further chemical purification, their serological properties were nevertheless such as to fulfil the requirements as reference antigens for the later studies The substances were kept at +4° C in an exsiccator

Antisera A system for the cross matching of type reference antigens and antisera was set up, following the pattern used in a previous series (*Omland* 1963c Fig 1) The result is shown in Fig 2

It is seen that the type systems a and f suffered from serious defects The a system yielded a comparatively weak homologous precipitation, while the strong heterologous precipitation (against f-antigen) indicated the presence of considerable amounts of non type-specific precipitin in the serum The f system yielded no, or very little, precipitation at all These shortcomings had to be eliminated before starting the examination of the material of collected strains

Type a Another experiment confirmed the finding of undesired quantities of non type-specific precipitin in serum no 3 In this experiment the heterologous type reference antigens were replaced by antigens prepared from 3 strains of the material described earlier (strains no 1, 2 and 4) These antigens proved richer in non-type-specific antigen than the type reference antigens thus being better indicators of corresponding non type specific precipitin in the serum

---

Fig 2 (please turn over)

Gel precipitation experiments showing type specific antigens and antisera in different homologous and heterologous combinations (see text)

Types	Symbols used for sera	Symbols used for type reference antigens
a	3	a
b	5	b
c	15	c
d	16	d
e	9	e
f	10 x	f

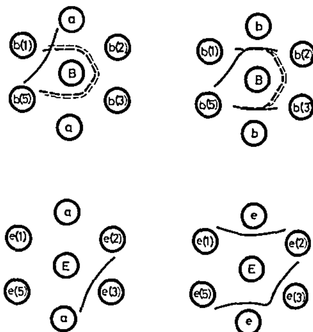


Fig 1

Antigen fractions prepared from type strains b 51 and e 51 in gel precipitation experiments against homologous antisera Identification of type specific antigen is shown in upper right experiment (type b antigen 1-volume fraction), and in lower right experiment (type e antigen, 3-volume fraction)

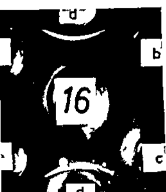
b (1) b (2) b (3) b (5) e (1) e (2) e (3) e (5)	b and e antigen fractions prepared by ethanol precipitation The volume proportions of ethanol are indicated in brackets
a b e	Antigens prepared from a 51 b 51, and e 51 by ethanol precipitation (5 volumes)

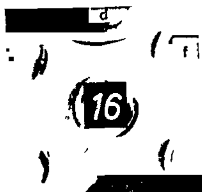
than 1 volume of ethanol for precipitation Consequently an ethanol precipitation using 1 1 5 volumes might be expected to yield the purest preparations of type specific antigens in types a, b, and c In type e, however, higher proportions of ethanol had to be employed (3 volumes) This particular e antigen appeared fortunately relatively free of non-type-specific elements, so that the higher proportion of ethanol could be used without great disadvantage

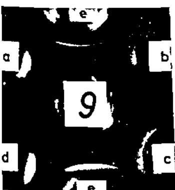
TABLE 1

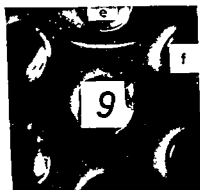
Preparation of Type Specific Reference Antigens for Gel Precipitation

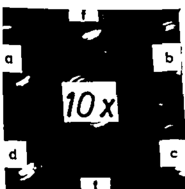
H influenzae type	Type strain used	Number of plates (chocolate agar) used for the preparation	Proportion of ethanol used for precipitation (vol)	Amount prepared (mg)
a	a 51	120	1 5	19 8
b	b 51	135	1 5	22 8
c	c 51	90	1 5	24 5
d	d 51	115	1 5	66 1
e	e 51	210	3 0	239 8
f	f 51	130	1 5	50 5
,	f D1	100	1 5	41 1











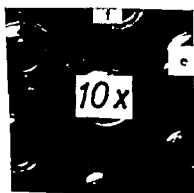
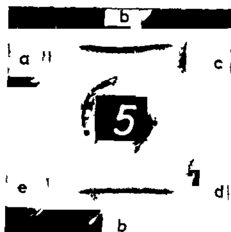
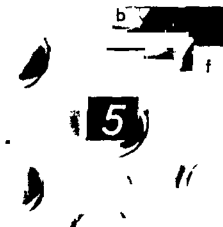


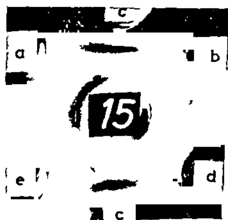
Fig 2











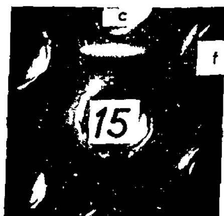


Fig 2

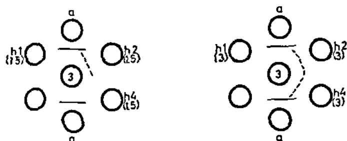


Fig 4

Gel precipitation experiments showing antigen fractions from strains no 1, 2, and 4 of the collected material against the a system in a gel to which has been added 0 05 per cent f II (see above)

h 1(1.5) h 1(3) h 2(1 5) h 2(3) h 4(1 5) h 4(3)	Antigen fractions prepared from strains no 1 2 and 4 of the collected material by ethanol precipitation The volume proportions of ethanol are indicated in brackets

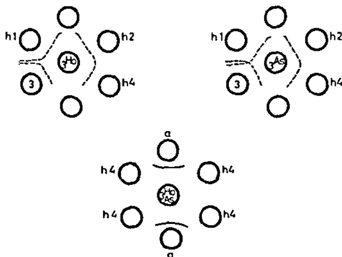


Fig 5

Experiments showing the effect of successive absorptions of serum no 3 by horse serum and ascitic fluid

h 1 h 2 h 4	Antigen fractions prepared from strains no 1, 2 and 4 of the collected material by precipitation with 1 5 volumes of ethanol
3	Unabsorbed serum no 3
3^{Ho}	Serum no 3 absorbed by horse serum
3^{As}	Serum no 3 absorbed by ascitic fluid
3^{Ho}_{As}	Serum no 3 absorbed by horse serum and ascitic fluid

As other available type a antisera were of still poorer quality, work was undertaken to improve the mentioned serum (serum no 3) This was done by absorption experiments Table 2 summarizes these experiments

TABLE 2
Absorption Experiments Performed on Serum No 3 (Type a)

Experiment no	Serum no 3 absorbed by	
	bacterial suspension (strain design)	soluble antigen (strain design)
1	h 1 (untypable)	
2	h 110 (untyp, rough)	
3	f 51	
4		f 51 (fraction fII)

Only one of the tried absorptions, *i e* the one employing material from type strain f 51 precipitated between the ethanol concentrations 1 5 and 5 volumes (the so called fraction fII) had any demonstrable effect on the non-type-specific precipitation The effect consisted in the removal of one of the lines, as shown in Fig 3

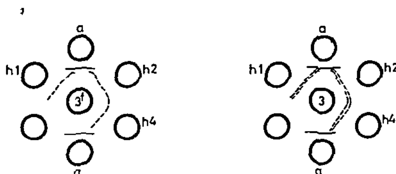


Fig 3

Experiments showing the effect of absorption of serum no 3 by an antigen fraction prepared from a heterologous type strain (f 51) One of the two demonstrable non type specific lines have been removed in the absorption

h 1 h 2 h 4	Antigen fractions prepared from strains no 1 2 and 4 of the collected material by precipitation with 1 5 volumes of ethanol
a	Control antigen prepared from a 51 by ethanol precipitation (5 volumes)
3^f	Serum no 3 absorbed by an antigen fraction prepared from f 51 by ethanol precipitation between 1 5 and 5 volumes (fraction f II)

These results were confirmed in an experiment where fII was added to the gel in a concentration of 0 05 per cent The result is shown in Fig 4 A comparison with Fig 3 reveals that most of the non type-specific precipitation has disappeared

values from 0 004 upwards in both absorbants All absorptions were carried out at +4° C for 2 days, and the precipitate was removed by high speed centrifugation The absorptions were performed successively, first with horse serum, thereafter with ascitic fluid

A repetition of the cross matching experiment (see Fig 2, first row), replacing the unabsorbed serum no 3 with doubly absorbed serum, showed that the non type specific precipitin had been removed without affecting the type specific antibody

Type f As seen from Fig 2 the f system consisting of serum no 10 x and reference antigen f yielded no precipitation The type strain f 51 was relatively slow growing, but also when older cultures (18 hours, instead of 4 6 hours) were used for animal inoculation satisfactory sera were not obtained Bacterial suspensions with the addition of Freund s adjuvant were tried without success

Finally a new type f strain (in this study designed fDi), kindly provided by dr Grace Leidy, New York, was used for inoculation With this strain several satisfactory sera were obtained The most potent one was serum no 47 which was used in the later work It might have been considered natural to use antigen prepared from the homologous strain (fDi) in the f system but as previous work had been done by means of antigen from f 51 (*Omland* 1963b c), it was found advisable to employ such antigen also in the further examinations A comparison was carried out between these two type f reference antigens against serum no 47 The fDi reference antigen had been prepared according to the same procedure as the f 51 antigen (see Table 1) The result of the comparison is shown in Fig 6

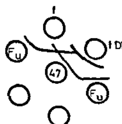


Fig 6

Experiment for the comparison of two type f reference antigens f from strain f 51 and fDi from strain fDi

f	f Di	Type reference antigens (see Table 1)
	47	Type f reference serum
	Fu	Type f reference serum kindly provided by dr Grace Leidy New York.

It is seen that the 47 fDi system contained at least two lines, while the 47f 51 system contained mainly one line, which however was sharp and distinct For reasons already mentioned it was then decided that

A priori two possibilities existed as to the origin of the antigens responsible for the non-type-specific phenomena. Either these antigens might be of bacterial nature or they might be components of the medium. The medium elements most likely to cause such effects were horse serum and human ascitic fluid, both contained in the heated horse blood agar used (*Omland* 1963c).

A portion of the serum (serum no. 3) was absorbed with 2 per cent horse serum, another with 2 per cent human ascitic fluid. A third portion was absorbed with both these substances successively in the said concentrations. Gel precipitations were carried out with the absorbed serum portions against the same bacterial antigens as those employed above (from strains no. 1, 2, and 4). The results are shown in Fig. 5.

Using unabsorbed serum no. 3 as a control (8-o'clock basins, upper row), excess absorbant was demonstrated in the respective absorbed serum portions. The lower photo shows that no demonstrable non-type specific precipitin was left in the serum after double absorption, using antigen from one of the mentioned strains (strain no. 4) as control.

It was considered undesirable in the later studies to use an absorbed serum containing too much excess absorbants. In order to avoid this gel precipitation experiments were set up to determine the "equivalence zones", keeping in mind the fact that the absorbants were both highly complex mixtures of antigens. Two series of absorptions were carried out, one with horse serum, the other with ascitic fluid. The following volumes of absorbants were used (quantity of serum no. 3 in all experiments 1 volume): 0.001, 0.002, 0.004, 0.006, 0.008, and 0.010. The results are indicated in Table 3.

TABLE 3
Titration Experiments for the Determination of "Equivalence Zones" in the Absorption of Serum No. 3 (Type a)

Absorbant	Absorbed serum in precip. against	
	*absorbant**	*unabsorbed serum§*
Horse serum	0.004	0.004
Ascitic fluid	0.008	0.004

* *Minimal concentration of absorbant for total absorption (parts per vol. of serum no. 3).*
§ *Minimal concentration of absorbant causing non precipitated excess (parts per vol. of serum no. 3).*

The final absorptions were performed on the basis of these results. In order to secure total absorption, volumes were chosen somewhat larger than the minimal values listed in Table 3 (left column), for horse serum 0.005, for ascitic fluid 0.010 (parts per vol. of serum no. 3). It was unavoidable under these conditions that absorbants were demonstrable in excess. Table 3 (right column) shows that this might be expected at

RESULTS

In no case was the interpretation of the gel precipitation difficult or doubtful Examples of positive results are shown in Fig 7 No type c *identification is shown*, as this type was not represented among the strains

It is remarkable that the ethanol precipitation characteristics of the e strains found in the collected material differ from the characteristics demonstrated in the type e reference strain (see above, Fig 1) All or most of the type specific antigen of the two e-strains diagnosed by aid of the gel precipitation method is precipitated by 1 5 volumes of ethanol In this respect these e-strains are thus similar to all other type strains examined in this study

The distribution of the different types in the material is presented in Table 5

TABLE 5

Distribution of H influenzae Strains According to Origin of the Pathological Material and Serotype as Determined by Gel Precipitation Tests

Origin	Serological type						Total no typable	Total no of strains	Per cent typable
	a	b	c	d	e	f			
Nose	4	15		3	1	5	28	95	29
Throat						1	1	3	33
Sputum		2					2	7	29
Bronchus				1			1	5	20
Sinus	1				1	1	3	24	12
Ear		4					4	10	40
Conjunctiva								2	
Vagina								1	
Cerebrosp fl		13					13	14	93
Blood		2					2	2	100
Totals	5	36		4	2	7	54	163	33

DISCUSSION

The initial part of the present study was concerned with the improvement and standardization of the type reference systems to be used in the investigation of the material of collected strains The gel precipitation method especially in the miniature modification described elsewhere (*Omland* 1963c), proved its versatility also in this type of work Thus it facilitated the selection of the serologically most active fraction of antigen, and in absorption experiments on sera it proved a valuable instrument *e g* in the determination of equivalence zones and the detection of excess antigen or antibody

In the actual task of surveying type specific antigens in a major material of strains the method *in the said modification*, was perhaps the only one feasible at the present time

the heterologous system 47/f51 should be employed in the subsequent studies of type specific antigens in the material of strains.

A final check on the type specificity of the new f-system was performed, using the cross-matching pattern shown in Fig 2.

The standard reference systems in all known serological types, as established by the work described, are listed in Table 4.

TABLE 4

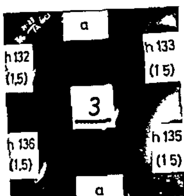
Type Reference Systems Used in Gel Precipitation Typing of H influenzae.

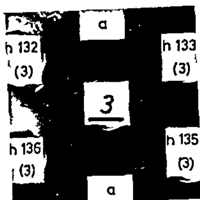
Type				Concentrations used in: first ab 10 strains typed	Concentrations used in: rest of material	Properties of type specific precipitation line
a	Serum	3	(abs)	undiluted	undiluted	weak,
	Antigen	a		1 mg/ml	2 mg/ml	slightly diffuse
b	Serum	5		undiluted	undiluted	strong,
	Antigen	b		0 1 mg/ml	0 1 mg/ml	sharp
c	Serum	15		undiluted	undiluted	strong,
	Antigen	c		0 5 mg/ml	0 2 mg/ml	sharp
d	Serum	16		undiluted	undiluted	strong
	Antigen	d		1 mg/ml	2 mg/ml	extremely sharp
e	Serum	9		undiluted	undiluted	strong,
	Antigen	e		1 mg/ml	2 mg/ml	extremely sharp
f	Serum	47		undiluted	undiluted	strong,
	Antigen	f		2 mg/ml	1 mg/ml	slightly diffuse

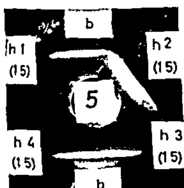
Examination of the Material of Collected Strains for Type Specific Antigens

The collection, classification, and certain serological examinations of the material of strains have been described previously (*Omland* 1963a, b)

The examination of this material for type specific antigens by means of the miniature modification of the gel precipitation (*Omland* 1963c) was performed in one series of experiments Each strain was inoculated directly from freezedried state (or via one subculture) onto a chocolate agar plate The plate was inoculated heavily and incubated for 2 days in a moist chamber before harvesting The preparation of soluble antigen was performed as described above (according to a modified *MacPherson's* procedure (1948)), yielding two fractions for each strain, one precipitated by 1 5 volumes of ethanol, the other precipitated between 1 5 and 3 volumes This was judged necessary because of the ethanol precipitation characteristics found in type e







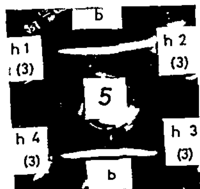


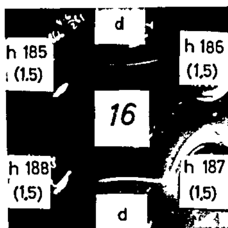
Fig 7

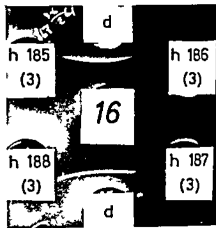
Examples of gel precipitation typing in types a b d e and f

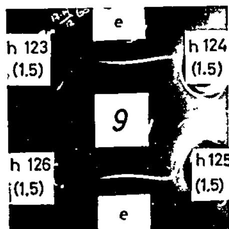
a b d e f	Type reference antigens (see Table 1)
3 5 16 9 47	Type reference sera (see Table 1)
h 132 h 133 etc	Antigen fractions prepared from strains no 132 133 etc The volume proportions of ethanol are indicated in brackets

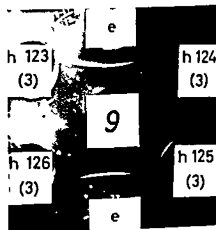
The distribution of serological types as registered by aid of this method is roughly similar to the distribution found by the capsular swelling procedure (*Omland* 1963b) however differing clearly in some respects In contrast to the high proportion of equivocal results registered by the capsular swelling method the gel precipitation method has yielded either affirmative or negative results There is a considerable difference in the incidence of the types h and e as determined by the respective methods

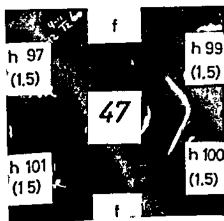
A comparison of the two sets of results with a view towards methodological and other problems will be presented in a subsequent article


d
h 185
(1,5)
h 186
(1,5)
16
h 188
(1,5)
h 187
(1,5)
d


d
h 185
(3)
h 186
(3)
16
h 188
(3)
h 187
(3)
d


e
h 123
(1,5)
h 124
(1,5)
9
h 126
(1,5)
h 125
(1,5)
e


e
h 123
(3)
h 124
(3)
9
h 126
(3)
h 125
(3)
e


f
h 97
(1,5)
h 99
(1,5)
47
h 101
(1 5)
h 100
(1,5)
f

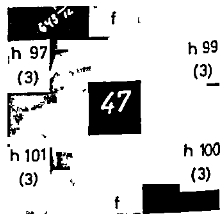
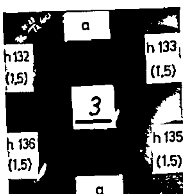
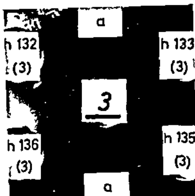
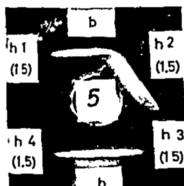

f
h 97
(3)
h 99
(3)
47
h 101
(3)
h 100
(3)
f

Fig 7







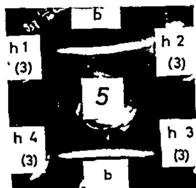


Fig 7

Examples of gel precipitation typing in types a b d e and f

a b d e f	Type reference antigens (see Table 1)
3 5 16 9 47	Type reference sera (see Table 1)
h 132 h 133 etc	Antigen fractions prepared from strains no 132 133 etc The volume proportions of ethanol are indicated in brackets

The distribution of serological types as registered by aid of this method is roughly similar to the distribution found by the capsular swelling procedure (*Omland* 1963b), however differing clearly in some respects In contrast to the high proportion of equivocal results registered by the capsular swelling method, the gel precipitation method has yielded either affirmative or negative results There is a considerable difference in the incidence of the types b and e as determined by the respective methods

A comparison of the two sets of results with a view towards methodological and other problems, will be presented in a subsequent article

SUMMARY

Work has been described with the purpose of bringing to perfection a set of type reference antigens and antisera to be used as standards in the serological typing of a collection of Haemophilus influenzae strains In this work as well as in the typing work itself the gel precipitation method has been employed, mostly as a miniature modification

Two fractions of antigen from each of the collected strains has been examined in gel precipitation experiments against known type specific systems The method has yielded clear cut results, as shown in Table 5 Among the 163 H influenzae strains 54 were typable The frequency distribution of the types was the following (by decreasing frequency) b, f, a, d, and e, thus differing somewhat from the corresponding result based on capsular swelling tests (*Omland* 1963b)

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SEROLOGICAL STUDIES ON HAEMOPHILUS INFLUENZAE AND RELATED SPECIES

5. *Evaluation of the Gel Precipitation and the Capsular Swelling Method by Comparison of the Two Sets of Results*

By

TOVE OMLAND

Received 22.ii.63

A previous article describes a material of Haemophilus strains collected from patients with various pathological conditions (*Omland* 1963a). For the purpose of serological characterization the material has been examined by means of the capsular swelling method (*Omland* 1963b), and a modification of the gel precipitation method (*Omland* 1963c).

The reason for using these two serological methods in parallel was to obtain as complete information as possible on the distribution of type specific antigens among the strains. The gel precipitation procedure in particular might be expected to yield detailed results in the study of such antigens. The parallel use of the two methods would clearly also give valuable data for the development of the modified gel precipitation technique into a tool for further serological studies of selected strains.

The employment of the two methods on the same material finally offered an opportunity for the assessment of each one in relation to the other. It is the purpose of the present article to report a comparison of the two sets of results, and on the basis of this comparison to discuss advantages and disadvantages of the methods in studies of this kind.

A direct correlation of the two sets of results is shown in Table 1.

Out of a total of 60 strains compared in the table only 32 have been identically characterized by the two techniques. If the doubtful results by the capsular swelling method are included, the number of equal typings is 39. Thus, in a very considerable number of cases the two methods yield contradictory results.

The degree of accordance of the two methods is illustrated in Table 2.

As a consequence of the arrangement the table contains 4 strains less than Table 1, i.e. those strains which were non-typable in gel precipitation and of doubtful type by capsular swelling. Out of a total of

TABLE 1

Correlation of Results as Determined by Capsular Swelling and Gel Precipitation Typings of a Material of H influenzae Strains

Serotype by capsular swelling	Serotype by gel precipitation a	b	c	d	e	f	Non typable	Totals
a	4(1)	(1)						4(2)
b		18(6)		(1)			(2)	18(9)
c							(1)	(1)
d		(1)		3				3(1)
e		2			2		2(1)	6(1)
f						5		5
nontypable		*8*				*2*		*10*
								46(14)
Totals	5	36		4	2	7	6	60

Numbers in brackets represent doubtful results by the capsular swelling method

TABLE 2

Degree of Accordance of Results of Gel Precipitation and Capsular Swelling Examinations in the Typing of a Material of H Influenzae Strains

	Identical type	Different type	Typable by gel precip Doubtful or non typ by capsular swelling	Typable by capsular sw Non typ by gel precip	Total
Number of results	32	2	20	2	56
Percentage	57	4	35	4	100

56 strains only 32 (57 per cent) have been identically characterized by the two methods (see also Table 1) The majority of the non-identical results consist of strains which were typable by gel precipitation, but non typable or of doubtful type by capsular swelling (20 strains or 35 per cent)

The outstanding fact revealed in these tables (Table 1 and 2) is the high incidence of doubtful results in the capsular swelling method Thus out of the 60 strains in Table 1 a total of 14 have been labelled as doubtful by the capsular swelling method It is also remarkable that as many as 8 out of these 14 strains are found among strains registered as type b by the gel precipitation method This is important because of the special clinical significance of that type

A comparison of the two sets of results as distributed among the various groups of pathological material is shown in Table 3

TABLE 3

Distribution of H influenzae Types According to Origin of the Pathological Material and Serotype as Determined by Capsular Swelling (Only Definitely Pos Results) and Gel Precipitation Tests

Origin	Serological type					Total no typable
	a	b	d	e	f	
Nose	3/4	8/15	2 3	3/1	4/5	20/28
Throat					0 1	0/1
Sputum		0 2				0/2
Bronchus			1/1			1/1
Sinus	1/1			1/1	1/1	3 3
Ear		2 4		1/0		3/4
Conjunctiva						
Vagina						
Cerebrospinal fl		7/13		1 0		8/13
Blood		1/2				1/2
Totals	4 5	18/36	3 4	6/2	5/7	36/54

No of *pos caps sw tests/no of pos gel pr tests*

One nose strain and one cerebrosp fl strain typed as e by caps sw but as b by gel pr

In this table only the unequivocal results attained by the capsular swelling method are included (36 strains) It is seen that the greatest discrepancies are found among type b and type e strains In type b only *one half of the strains labelled as such by the gel precipitation method* have been registered by the capsular swelling procedure In type e on the contrary a greater number of strains have been detected by the capsular swelling method than by gel precipitation This should be kept in *mind while attention is drawn to the unique position of type e* as revealed from an entirely different angle *viz* as concerns the ethanol precipitation characteristics of the type specific antigen (*Omland* 1963c) Further investigations on the problems of type e will be reported in a subsequent study

Examining the groups of pathological material in Table 3 it is noted that the capsular swelling technique has failed to register nearly one *half* of the type b strains found in cerebrospinal fluid This is a reflection of what was stated above concerning type b

In interpreting these comparisons of results a reservation must be made on the ground of the slight difference in reference systems used in the respective methods From the detailed reports of the investigations made by each method (referred to above) it is seen that during the gel precipitation studies another type f serum has been used than in the other method (serum no 47 instead of serum no 10 x) Finally the type a serum (serum no 3) has been absorbed before use in the gel precipitation experiments

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b		18(6)		(1)			(2)	18(9)
c							(1)	(1)
d		(1)		3				3(1)
e		2			2		2(1)	6(1)
f						5		5
nontypable		8				2		10
								46(14)
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The outstanding fact revealed in these tables (Table 1 and 2) is the high incidence of doubtful results in the capsular swelling method. Thus out of the 60 strains in Table 1 a total of 14 have been labelled as doubtful by the capsular swelling method. It is also remarkable that as many as 8 out of these 14 strains are found among strains registered as type b by the gel precipitation method. This is important because of the special clinical significance of that type.

A comparison of the two sets of results as distributed among the various groups of pathological material is shown in Table 3.

TABLE 3

Distribution of H influenzae Types According to Origin of the Pathological Material and Serotype as Determined by Capsular Swelling (Only Definitely Pos Results) and Gel Precipitation Tests

Origin	Serological type					Total no typable
	a	b	d	e	f	
Nose	3/4	8 15	2/3	3/1*	4/5	20 28
Throat					0/1	0/1
Sputum		0 2				0/2
Bronchus			1/1			1/1
Sinus	1/1			1/1	1/1	3 3
Ear		2/4		1/0		3/4
Conjunctiva						
Vagina						
Cerebrospinal fl		7/13		1/0*		8 13
Blood		1/2				1/2
Totals	4 5	18 36	3 4	6 2	5/7	36/54

No of pos caps sw tests/no of pos gel pr tests
* One nose strain and one cerebrosp fl strain typed as e by caps sw but as b by gel pr

In this table only the unequivocal results attained by the capsular swelling method are included (36 strains) It is seen that the greatest discrepancies are found among type b and type e strains In type b only one half of the strains labelled as such by the gel precipitation method have been registered by the capsular swelling procedure In type e, on the contrary, a greater number of strains have been detected by the capsular swelling method than by gel precipitation This should be kept in mind while attention is drawn to the unique position of type e as revealed from an entirely different angle, *viz* as concerns the ethanol precipitation characteristics of the type specific antigen (*Omland* 1963c) Further investigations on the problems of type e will be reported in a subsequent study

Examining the groups of pathological material in Table 3 it is noted that the capsular swelling technique has failed to register nearly one half of the type b strains found in cerebrospinal fluid This is a reflection of what was stated above concerning type b

In interpreting these comparisons of results a reservation must be made on the ground of the slight difference in reference systems used in the respective methods From the detailed reports of the investigations made by each method (referred to above) it is seen that during the gel precipitation studies another type f serum has been used than in the other method (serum no 47 instead of serum no 10 x) Finally the type a serum (serum no 3) has been absorbed before use in the gel precipitation experiments

DISCUSSION

Two techniques as different as the gel precipitation and the capsular swelling method must necessarily cover different areas of application. Generally speaking the gel precipitation is an objective immunochemical method, while the capsular swelling method through its direct microscopical observation approaches the cytological methods.

The advantages and disadvantages of the two methods may be briefly summarized as follows.

	Advantages	Disadvantages
Gel precipitation	Exactness Reproducibility Semi-quantitative features	Relative slowness Need of greater amount of antigen Risk of denaturation of antigen Need of relatively pure reference systems
Capsular swelling	Rapidness Results partly in cytological terms *(localization of antigen)*	*Subjectivity in observation* Difficulty in observation in small organisms

In an investigation like the present study of H. influenzae certain of these points are especially relevant. Concerning the gel precipitation, all the advantages would clearly be of great value. The disadvantages have been partly eliminated in the miniature modification of the method, thus particularly the slowness and the need of great amounts of antigen, but also the need of relative pure reference systems. A significant step towards removing unspecific elements from the systems have been made by absorption experiments. Concerning the capsular swelling method the advantages mentioned are of little importance in this type of studies, whereas the disadvantages retain their full significance. In this connection H. influenzae must be characterized as a "small organism" compared to *e.g.* the pneumococcus.

In addition to a general methodological discussion it would be of *special interest to assess the relative value of the two methods* on the basis of the results as presented in the tables above, particularly Table 2. It must be assumed that the positive results by gel precipitation are indisputable, as they are based on identity (or fusion) reactions with reference antigens. The large group containing strains positive by gel precipitation and negative or doubtful by capsular swelling reactions must consequently represent a deficiency in the latter method. The small group (2 strains or 4 per cent) containing definitely different results of typing likewise appears to be due to failures in the capsular swelling method.

These comments pertain to the modification of the capsular swelling *method* as practised by the author, a modification in which precautions had been taken to secure a favourable performance (*Omland 1963b*).

A small group remains consisting of 2 strains (4 per cent) which were positive in capsular swelling tests but negative by gel precipitation Keeping in mind the general methodological drawbacks of the capsular swelling technique, and noting especially the occurrence of a large proportion of doubtful results it is natural to suspect these findings of being false positive reactions There is however, the obvious possibility that the precipitation antigens may have become inactive in some way As seen from Table 1 the 2 strains are typed as e strains by capsular swelling reactions and as already mentioned problems concerning this type will be taken up in a subsequent study

It has been pointed out earlier that the reference systems in the two procedures have not been exactly the same Certain changes have been necessary in type a and type f In spite of this, complete correlation exists between the two sets of results in type f, and except for 1 strain also in type a (see Table 1) It is thus highly improbable that this point has played a role which would make a comparison of methods and results less admissible

the gel precipitation technique
-d its superiority over the cap-
on type specific H influenzae
antigens

SUMMARY

Two sets of results of serological studies on H influenzae type specific antigens have been correlated to each other One set has been registered by aid of the gel precipitation method the other by the capsular swelling method Based on this comparison and on general methodological viewpoints it is concluded that the gel precipitation method is superior in this type of work

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FROM THE INSTITUTE OF FORENSIC MEDICINE, UNIVERSITY OF OSLO RIKSHOSPITALET, OSLO, NORWAY

A HETEROGENEITY OF THE FAST MOVING COMPONENT OF THE Gc-SYSTEM

By

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Received 29 iv 63

The Gc-system was discovered by *Hirschfeld* in 1959 (5) by the use of a modified immuno-electrophoretic technique (3, 13, 6) The distinction between the different types within the Gc-system is based on the appearance in the immuno-electrophoretic slides of an α_2 globulin, the physiological function of which is still unknown By Hirschfeld's technique, Gc type 1–1 appears as a single arc nearer to the anode, type 2–2 *is a single arc nearer to the cathode;* while type 2–1 *appears as a* prolonged, flattened or double-peaked arc, which is identical to the picture given by a mixture of equal parts of sera of the two other types

The inheritance of the Gc types has been established by the study of large family materials and numerous mother-child combinations (9, 1, 2, 12, 7, 4, 10)

By Hirschfeld's technique, the great majority of human sera give typical Gc precipitation patterns according to one of the three types described above Aberrant types are very rare (7, 8) Based on the typical findings, it would be logical to assume that the genes Gc^1 *and* Gc^2 (whether inherited in single or double dose) express themselves as homogeneous serum protein factors of fast and slow mobility respectively, and that the two components are immunologically identical as defined by the animal antisera used

The purpose of this study is to present evidence for a heterogeneity of the fast moving group specific component, while the slow moving component appears homogenous by the same method of analysis The investigation is based upon a modified immuno-electrophoretic technique, developed for this purpose The principle characteristic of this technique is a prolonged electrophoretic separation time

MATERIALS AND METHODS

Human sera Blood was collected by venepuncture allowed to clot for two hours at 37° C and overnight at 4° C Serum was then decanted cellular elements removed by centrifugation and the serum was kept frozen at —25° C until used In all preliminary experiments including those presented in Fig 2 serum was tested within a week after the bleeding of the the donor

Antisera Antisera were obtained from rabbits immunized with pooled human serum by Proom's method (11) Each rabbit was given two or more immunization series For the modified technique, antisera were selected which gave particularly firm and fast appearing Gc precipitates

Staining of bovine albumin Bovine albumin 30 per cent (Armour Pharmaceutical Company Ltd) was diluted to 4 per cent with distilled water Amido Black 10 B (George T Gurr Ltd) was added to a final concentration of approximately 1/100 (w/v) and the solution was dialysed overnight against running tap water The stained bovine albumin was used as a visible marker of electrophoretic migration velocity (10) Bovine albumin was preferred to human as it did not form precipitates with the rabbit antihuman sera used The electrophoretic migration of the stained bovine albumin was slightly faster than that of the albumin of whole human serum

Agar Difco Bacto Agar (Control No 452925) was prepared according to *Hirschfeld* (6)

Buffers Barbital-calciumlactate buffers of pH 8 6 were prepared as described by *Hirschfeld* (6) for electrode compartments and for mixing with the agar

Filter paper A double layer of Whatman filter paper No 3 moistened with buffer was used for electric connection between electrode buffer compartments and the agar slides

Photographic registration After the development of the precipitation patterns all slides were photographed in moist unstained condition using indirect light A Leica camera and 35 mm Agfa Agepe film were used

The Modified Immuno-Electrophoretic Procedure

Glass slides (12 × 12 cm) were carefully cleaned and defatted with alcohol-ether Placed in a horizontal position, they were evenly covered with 21 ml of a molten 1 per cent agar solution in barbital-calcium-lactate buffer pH 8 6 with merthiolate added to a final concentration of 1/10 000 (w/v) The thickness of the agar layer was 1 5 mm When the gel had solidified, circular holes were punched out with a pipette and the contours of a longitudinal basin prepared with a double knife as shown on Fig 1

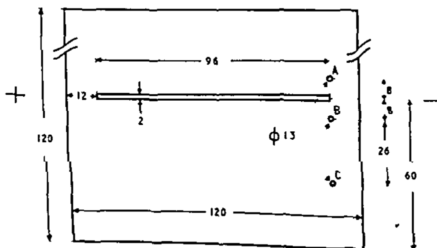


Fig 1

Dimensions of the slide and of the basins in the gel layer used for the modified immuno-electrophoretic technique All measures are in millimeters

The circular wells A and B were filled with 2 microlitres of the human sera to be tested by means of Carlsberg pipettes, and well C was filled with stained bovine albumin solution The two ends of the agar slide were connnected with the electrode buffer by filter paper moistened with the same buffer The serum proteins were then subjected to electrophoresis at approximately 6 V/cm until the easily visible centre of the stained bovine albumin spot had migrated 9 cm towards the anode The process was completed in approximately 5 hours The centre of the albumin spot of human serum had then migrated 8 cm The agar strip between the contours of the longitudinal basin was removed, and the basin was filled with 100 microlitres of antiserum The slide was placed horizontally in a moist chamber at 37° C, and the precipitation patterns were read daily The Gc precipitates had usually reached their maximal density after 48 hours, and they were then photographed

Methodological Considerations

It was possible to examine more than two sera in parallel runs on the samé slide With the equipment used, however, evaporation from the sides of the gel layer could not be entirely avoided during the prolonged electrophoresis Strictly comparable conditions were obtained only by restricting the analysis to two sera, located symmetrically on both sides of the middle line on each slide

Because of the evaporation, the great width of the slides was considered to be an advantage

When the albumin spot of human serum had migrated 8 cm towards the anode, conditions were optimal for the purpose of this study Extension of the electrophoresis beyond this point offered no advantage

RESULTS

Three normal human sera, representing the three common Gc-types by the conventional method (see Fig 2, upper part), were examined by the modified technique The results are shown in Fig 2, lower part

The serum of Gc type 1 1 produced a clearly double-peaked arc in the anodic part of the Gc-area Gc 2–1 appeared as a prolonged triple peaked arc Gc 2–2 remained as a single symmetrical arc in the cathodic region

So far, the sera from 40 adult, unrelated persons have been investigated by this method Of these, 20 were of Gc type 1 1, 15 of type 2–1, and 5 of type 2–2 The Gc protein in the sera from all the different persons of Gc type 2 2 produced a single symmetrical arc, while the fast moving component in all sera of type 1 1 and 2 1 showed double-peaking

The two peaks formed by the fast moving component varied in length thickness and in their distance from the antibody basin The degree of these variations was *different among the individuals* When the varia-

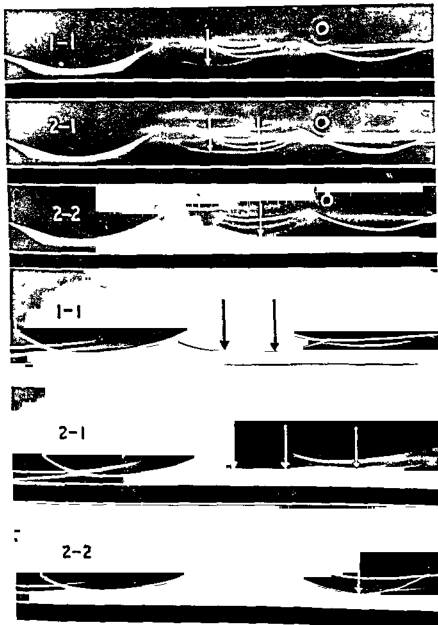


Fig. [illegible]

Typical precipitation patterns of three normal human sera belonging to the three common Gc types. Immuno electrophoretic examination with Hirschfeld's technique (upper part) and with the modified technique (lower part). The peaks of the Gc precipitates are indicated by arrows. Two different antisera, each giving optimal conditions for the different techniques, have been applied in the tests.

tions were pronounced, the cathodic peak was always the longest, thickest and the closest to the antibody basin In some sera, as in those of types 1–1 and 2–1 shown in Fig 2, no difference between the two peaks could be detected The pattern of each individual's serum was reproduced, within narrow limits, in different tests, and samples taken at various times from the same donor always showed the same result

The individual shapes of the fast Gc precipitate showed gradual transitions from one form to the other, and, accordingly, no clear distinction between subtypes within the Gc-system was possible The study of a small family material failed to give evidence that the precipitation shape of the fast moving component was genetically determined

DISCUSSION

By the use of a modified immuno-electrophoretic technique, it has been shown that the fast moving group specific component (Gc 1) probably is heterogenous The findings described are most likely explained if Gc 1 were assumed to consist of two different kinds of protein molecules with slightly different electrophoretic mobility and with immunological identity as defined by the antisera used Thus the presence of both these proteins has to be regarded as determined by the gene Gc^1 The different individual variations within the Gc 1 precipitate indicate that the concentration ratio between the two proteins may differ from equality to an overweight of the slower moving variety

Antisera producing rapidly appearing Gc precipitates gave the clearest picture of the double-peaked Gc 1 arcs This was probably due to the fact that the two sub-components now were precipitated before they had a chance to diffuse into one single spot

It is not likely that the phenomenon described has anything to do with the aberrant group specific components X or Y (7, 8) These aberrant factors are very rare, and the heterogeneity of Gc 1 was present in all sera of types 1 1 and 2–1 so far examined However, no sera of aberrant Gc types have been available for studies with the modified technique

As reproducible differences within the Gc 1 precipitate have been detected, the possibility of inheritable Gc sub types has to be considered No evidence for the genetic control of the individual variations of Gc 1 has been found in the study of a small family material, but more extensive studies are needed to solve this problem

It seems improbable that the heterogeneity of Gc 1 is due to the action of two Gc^1-genes parallel to Hp^{1F} and Hp^{1S} as Smithies et al have discovered in the Hp-system (16) The Gc 2 1 precipitate should then only contain either the fast or the slow Gc 1 peak not both as was found in all Gc 2–1 sera so far examined

Cleve & Bearn (2) observed reproducible individual differences in the shape of Gc 1–1 and 2–1 precipitates This may be the same phenomenon

as described in this paper, but their description is not detailed enough to be used for comparison

Smithies (15) has described individual variations in the post-albumin region by means of vertical starch gel electrophoresis He states that other "post-albumin types" have been observed in addition to the three illustrated in his paper *Schultze et al* (14) have demonstrated a probable identity between the post-albumins of Smithies and the group specific components of Hirschfeld It is possible that the individual differences within the fast moving group specific component is the explanation of Smithies's finding of more than three different post-albumin types

One has to consider the possibility that the heterogenity is an artefact due to cleavage of native protein molecules during the prolonged electrophoresis The homogeneity of the slow moving component seems to contradict such a hypothesis If the migration of the slow moving component is extended to a point where Gc 1 clearly presents a double-peaked arc, *it still remains as a symmetrical single arc* The possibility exists, however, that the fast moving component is less stable under these conditions No similar changes in the precipitates formed by other proteins have been noticed in the preparations

SUMMARY

A modified immuno-electrophoretic technique has been applied in the study of the Gc types of Hirschfeld The main difference from the conventional method is a prolonged electrophoretic separation time By this technique a heterogeneity of the fast moving group specific component has been detected, which was present in all of 35 sera of Gc types 1–1 and 2 1 so far examined Sera of Gc type 1–1 produced a double-peaked precipitate and those of Gc type 2–1 a triple peaked precipitate The slow moving group specific component appeared homogenous by the same method of analysis in all of 5 sera examined

The significance of these findings are discussed In the author's opinion, the results are most likely explained if Gc 1 were assumed to consist of two different proteins with slightly different electrophoretic mobility and that both are determined by the Gc^1-gene

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POLYVALENT, DIAGNOSTIC PNEUMOCOCCUS SERA

By

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In pneumococcal infections, particularly in menigitis, it is important to know the bacteriological diagnosis quickly in order to start effective treatment at once. It is possible, by means of Neufeld's capsular reaction, to identify pneumococci present in a specimen within a few minutes, but as meningitis may be caused by any type the test should be done with pooled serum reacting with all types of pneumococcus. It is not reliable if done, as has been proposed, with serum containing only the most common types. Statens Seruminstitut has produced three kinds of diagnostic Pneumococcus sera (*Lund* 1960):

(1) *Forty six type or group sera,* which react with single types or groups, numbered 1–48, the numbers 26 and 30 are not used. The earlier types 26 and 30 have been changed to the types 6B and 15A (Table 1).
(2) *Nine pooled sera,* each reacting with 7–11 types, together covering the 81 known Pneumococcus types (Table 2).
(3) *One omni-serum,* omnivalent, reacting with all 81 types.

The diagnostic Pneumococcus sera are meant for Neufeld's capsular reaction and not for agglutination tests. The reaction of the capsule is not a swelling but an optical change giving a visible outline. The added methylene blue colours the body, but not the capsular substance.

The capsular titres of the diagnostic sera will by delivery be as follows:

type and groups serum	≥ 32
pooled serum	≥ 16
omni serum	≥ 8

Some of the type and group sera have strong antibodies causing cross-reactions with other types, but would be too weak if these antibodies were absorbed. The following three sera are not type-specific:

Serum Pn 29	reacts with type 35B
Serum Pn 35	reacts with types 29, 47
Serum Pn 42	reacts with types 35A, 35B, 35C

TABLE 1

Capsular Antigens of the Pneumococci (81 Types).

Type	Antigenic formulas	Type	Antigenic formulas
1	1a	21	21a
2	2a	22	22a, 22b
3	3a	22A	22a, 22c
4	4a	23	23a, 23b, 18b
5	5a	23A	23a, 23c, 15a
6A	6a, 6b	23B	23a, 23b, 23d
6B	6a, 6c	24	24a, 24b, 24d, 7h
7	7a, 7b	24A	24a, 24c, 24d
7A	7a, 7b, 7c	24B	24a, 24b, 24e, 7h
7B	7a, 7d, 7e, 7h	25	25a, 25b
7C	7a, 7d, 7f, 7g, 7h	27	27a, 27b
8	8a	28	28a, 28b, 16b, 23d
9A	9a, 9c, 9d	28A	28a, 28c, 23d
9L	9a, 9b, 9c, 9f	29	29a, 29b, 13b
9N	9a, 9b, 9e	31	31a, 20b
9V	9a, 9c, 9d, 9g	32	32a, 27b
10	10a, 10b	32A	32a, 32b, 27b
10A	10a, 10c, 10d	33	33a, 33b, 33d
11	11a, 11b, 11e, 11g	33A	33a, 33b, 33d, 20b
11A	11a, 11c, 11d, 11e	33B	33a, 33c, 33d, 33f
11B	11a, 11b, 11f, 11g	33C	33a, 33c, 33e
11C	11a, 11b, 11c, 11d, 11f	34	34a, 34b
12	12a, 12b	35	35a, 35b 34b
13	13a, 13b	35A	35a, 35c, 20b
13	14a	35B	35a, 35c, 29b
14	14a	35C	35a, 35c, 20b, 42a
15A	15a, 15c, 15d, 15g	36	36a, 9e
15B	15a, 15b, 15d, 15e, 15h	37	37a
15C	15a, 15d, 15e	38	38a, 25b
16	16a, 16b, 11d	39	39a, 10d
17	17a, 17b	40	40a 7g, 7h
17A	17a, 17c	41	41a, 41b
18	18a, 18b, 18c 18f	41A	41a
18A	18a 18b, 18d	42	42a 20b, 35c
18B	18a, 18b, 18e, 18g	43	43a
18C	18a, 18b, 18c, 18e	44	44a 20b 12b
19	19a, 19b 19d	45	45a
19A	19a 19c, 19d	46	46a 44b
19B	19a, 19c 19e 7h	47	47a, 35a 35b
19C	19a, 19c, 19f 7h	48	48a
20	20a 20b 7g		

TABLE 2

Pooled Diagnostic Pneumococcus Sera

Pool	Reacting with the types or groups	Immunized with the types
A	1-2-4 5 18	1 2 4 5 18 18A 18B 18C
B	3-6-8-19	3 6A 6B 8 19 19A 19B 19C
C	7-20-24-31 40	7 7A 7B 7C 20-24 24A 24B 31 40
D	9-11 16-36-37	9A 9L 9N 9V 11 11A 11B 11C 16 36 37
E	10-12 21 33 39	10 12 21 33 33A 33B 33C 39
F	17-22-27 32 41	17 22 22A 27 32 32A 41 41A
G	29-34 35-42 47	29 34-35 35A 35B 35C 42 47
H	13-14-15 23-28	13 14-15 15A 15B 15C 23 23A-23B 28 28A
I	25-38-43-44-45 46 48	25 38-43 44 45 46 48

Type 2 Pneumococcus serum is absorbed with vaccine of Klebsiella type 2 (formerly called B Friedlander), but no other Pneumococcus serum has been absorbed with any other organisms than pneumococci Common antigens have however been described between pneumococci and many other bacteria (Salmonella Klebsiella E coli B anitratum Haem influenzae non haemolytic streptococci etc) To make sure that an organism is a Pneumococcus it is necessary in addition to the Neufeld test to test its sensitivity to optochin (*Lund* 1959) and to study its morphology microscopically and in cultures

Pneumococci may be distinguished from streptococci by the optochin test R and S pneumococci are both sensitive for optochin but in an India ink preparation an S Pneumococcus will show a capsule of the same size as in a Neufeld reaction

The omni serum is intended for quick diagnosis especially in cerebro spinal fluids It is recommended that an examination with a type 3 serum is always included as the diagnosis of this type presents certain difficulties because the capsules autolyse very quickly

Omni serum is produced by mixing equal parts of the most potent pooled sera A I In order to raise the titres omni serum is concentrated to about half the amount by salting out the γ globulins Alternatively it may be freeze dried and redissolved in a smaller amount of fluid (aqua dest) These two methods may be combined

The diagnostic sera are usually kept at +4° C Experimentally certain amounts of the pooled sera A I were placed at —22°C Some of these sera (pool F and G) did not stand this treatment After a few months the sera become semisolid and they did not liquify even after several days at 37 C These sera gave very weak capsular reactions the outlines not being clearly visibly anymore

It has been found that non absorbed Pneumococcus sera keep better than the corresponding absorbed sera The more a serum has been absorbed the shorter the time it keeps As the diagnostic sera are absorbed to different extents there are no exact rules for their life The type- and group sera and the pooled sera are usable for at least two years when kept at + 4° C but in most cases these sera keep much longer to date omni serum has been unchanged after 12 months at +4° C

Bacterial meningitis is caused most often by *meningococci* pneumo cocci and Haemophilus influenzae (*Rosendal* 1955) To differentiate pneumococci from H influenzae in cerebro spinal fl

Diagnostic serum for H Influenzae is at present only produced at Statens Seruminstitut for type b This serum may react with certain types of pneumococci but as the diagnostic H influenzae—sera examined up to now have been of rather low titres antibodies to pneumo cocci have not been absorbed In a concentrated serum the number of

TABLE 1

Capsular Antigens of the Pneumococci (81 Types)

Type	Antigenic formulas	Type	Antigenic formulas
1	1a		
2	2a	21	21a
3	3a	22	22a 22b
4	4a	22A	22a 22c
5	5a	23	23a 23b 18b
6A	6a 6b	23A	23a 23c 15a
6B	6a 6c	23B	23a 23b 23d
7	7a 7b	24	24a 24b 24d 7h
7A	7a 7b 7c	24A	24a 24c 24d
7B	7a 7d 7e 7h	24B	24a 24b 24e 7h
7C	7a 7d 7f 7g 7h	25	25a 25b
8	8a	27	27a 27b
9A	9a 9c 9d	28	28a 28b 16b 23d
9L	9a 9b 9c 9f	28A	28a 28c 23d
9N	9a 9b 9e	29	29a 29b 13b
9V	9a 9c 9d 9g	31	31a 20b
10	10a 10b	32	32a 27b
10A	10a 10c 10d	32A	32a 32b 27b
11	11a 11b 11c 11g	33	33a 33b 33d
11A	11a 11c 11d 11e	33A	33a 33b 33d 20b
11B	11a 11b 11f 11g	33B	33a 33c 33d 33f
11C	11a 11b 11c 11d 11f	33C	33a 33c 33e
12	12a 12b	34	34a 34b
13	13a 13b	35	35a 35b 34b
13	14a	35A	35a 35c 20b
14	14a	35B	35a 35c 29b
15A	15a 15c 15d 15g	35C	35a 35c 20b 42a
15B	15a 15b 15d 15e 15h	36	36a 9e
15C	15a 15d 15e	37	37a
16	16a 16b 11d	38	38a 25b
17	17a 17b	39	39a 10d
17A	17a 17c	40	40a 7g 7h
18	18a 18b 18c 18f	41	41a 41b
18A	18a 18b 18d	41A	41a
18B	18a 18b 18e 18g	42	42a 20b 35c
18C	18a 18b 18c 18e	43	43a
19	19a 19b 19d	44	44a 20b 12b
19A	19a 19c 19d	45	45a
19B	19a 19c 19e 7h	46	46a 44b
19C	19a 19c 19f 7h	47	47a 35a 35b
20	20a 20b 7g	48	48a

TABLE 2

Pooled Diagnostic Pneumococcus Sera

Pool	Reacting with the types or groups	Immunized with the types
A	1 2 4 5 18	1 2 4 5 18 18A 18B 18C
B	3 6 8 19	3 6A 6B 8 19 19A 19B 19C
C	7 20–24 31 40	7 7A 7B 7C 20–24 24A 24B 31 40
D	9 11 16 36 37	9A 9L 9N 9V 11 11A 11B 11C 16 36 37
E	10 12 21 33 39	10 12 21 33 33A 33B 33C 39
F	17 22 27 32 41	17 22 22A 27 32 32A 41 41A
G	29 34 35 42 47	29 34 35 35A 35B 35C 42 47
H	13 14–15 23 28	13 14 15 15A 15B 15C 23 23A 23B 28 28A
I	25 38–43 44 45 46 48	25 38 43 44 45 46 48

FROM THE INSTITUTE OF VIROLOGY AND THE INSTITUTE OF BACTERIOLOGY
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THE AETIOLOGY OF RESPIRATORY TRACT INFECTIONS IN MILITARY PERSONNEL

3 The Recovery of ECHO 28 from Cases with Minor Respiratory Illness

By

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Received 29 v 63

Some enteroviruses have been associated with respiratory disease Coxsackie A21 and ECHO 28 have both been implied as causative agents of minor respiratory illness in military recruits in several studies (1 5)

The present report is concerned with an endemic outbreak of minor respiratory illness in military recruits continuously studied for the aetiology of respiratory infections An association between infection with ECHO 28 virus and the disease is demonstrated

MATERIAL AND METHODS

The investigation was carried out in a military camp at Uppsala according to plans previously described in detail (6–8) Only conscripts reporting for respiratory disease at the hospital of the camp were examined More detailed information about the techniques used has been reported earlier (6–8)

Collection of Specimens

Oropharyn
0 5
not
anc
ta r
2° C until tested

Virus Isolation

) days Haem
cultures

Virus Identification

Cytopathogenic agents were identified by *neutralization* with specific antisera against the different viruses A rabbit serum against ECHO 28 as well as the prototype strain of this virus were kindly supplied by Dr W J *Mogabgab* New Orleans U S A

antibodies to pneumococci will be higher than in the natural serum, so *for diagnostic purposes the use of natural serum is recommended*

SUMMARY

Statens Seruminstitut has produced three kinds of diagnostic Pneumococcus sera

1) 46 type or group sera, 2) 9 pooled sera together covering all 81 types and 3) an omni serum, reacting with all types

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effect was most readily observed if young cultures with islands of cells were inoculated instead of outgrown monolayers In infected KB cultures all the cells became degenerated

Serological Findings

Sera from all 54 cases were investigated for complement-fixing and haemagglutination-inhibition antibodies against several viral antigens as described in Methods Table 2 shows the results obtained when the sera were tested for neutralizing antibodies against ECHO type 28 both in cynomolgus monkey kidney and KB cell cultures Ten cases had fourfold or greater increase in antibody titres when tested in KB cultures, the same 10 cases showed fourfold increase when tested in monkey kidney cultures Fifteen additional cases had high antibody titres in all three sera tested in each case

TABLE 2

Demonstration of Antibodies against ECHO 28 Virus by two Different Techniques

Total number of cases	Number of cases with fourfold increase when tested in monkey kidney cultures	Number of cases with fourfold increase when tested in KB-cell cultures	Additional cases with antibody titres above 1 64
54	10	10	15

A fourfold increase in complement-fixing and HI-antibodies against parainfluenza type 3 virus was demonstrated on the paired sera from the case with an isolate of this virus High titres of complement-fixing antibodies against adenovirus were observed in all three sera from the case with an isolate of adenovirus type 2 No significant titre increases were observed in the tests with other antigens

When the cases with a fourfold increase in antibody titre against ECHO 28 virus and the cases with high antibody titres in all paired sera were plotted against time of onset of illness, it appears that at least two waves of ECHO 28 infection occurred in the camp, as seen in Fig 1 The first incidence started at January 12 and the second at January 31

Bacteriological Findings

The results of the bacteriological examination as well as significant titre rises against bacterial antigens are shown in Table 3 Bacterial infections did apparently not contribute to the clinical disease observed

Clinical Findings

The conscripts reporting for respiratory disease were examined by a physician in the team and symptoms and signs recorded The main symptoms were coryza, cough, sore throat and malaise The signs

Bacteriological Technique

The specimens were investigated according to techniques previously described (7) Potential pathogens of the respiratory tract as *Staphylococcus aureus* haemolytic streptococci *Haemophilus influenzae* and pneumococci were recorded

Serology

Neutralizing antibodies were determined by incubating equal amounts of serial twofold dilutions of serum and 100 TCD$_{50}$ of virus for 1 hour at 37° C The mixtures were inoculated in monkey kidney or KB cultures which were finally read on day 5 and day 3 respectively after inoculation KB cells were propagated in Eagles minimum essential medium (MEM) with 10 per cent calf serum In neutralization tests the maintainance medium was 2 per cent inactivated (56° C 30 min) chicken serum in Eagles MEM Complete suppression of the cytopathic effect was used in determining endpoints

Complement fixation tests were carried out in tubes with 0.1 ml of antigen and serum respectively, according to a method previously described (6) All sera from the diseased conscripts were examined for complement fixing (CF) antibodies against adenoviruses influenza A B and C viruses mumps and parainfluenza virus type 1-3 herpes simplex and ornithosis Haemagglutination inhibition (HI) tests were also performed against parainfluenza virus type 1-3 Coxsackie A21 and ECHO 19 according to methods previously described (6, 9) The preparation of antigens has been described in a previous communication (6)

Antibacterial antibodies antistaphylolysin, antistreptolysin and antipneumolysin were determined according to methods previously described (7) Complement fixing antibodies against *H influenzae* were determined with a sonicated extract of whole bacteria as the antigen

RESULTS

Virus Isolation

During the period January 15 February 21, 1962, 54 recruits of a total of 710 conscripts reported for respiratory disease at the hospital of the military camp From two cases ECHO virus type 28 was isolated from the first throat specimen, as seen in Table 1 In another case adenovirus type 2 and finally a strain of Parainfluenza virus type 3 was recovered from one recruit, as seen in Table 1

TABLE 1
Virus Isolations in Military Recruits with Respiratory Disease

Total number of cases	Number of cases with isolates of			
	ECHO 28		Adenovirus type 2	Parainfluenza virus type 3
	Throat	Faeces		
54	2	0	1	1

At isolation the ECHO 28 virus only showed cytopathic effects in the margin of the monkey kidney cell cultures Cytopathic effect was first observed 3 days after inoculation and was more pronounced when the cultures were incubated at 33° C As a rule virus yields of 10^{4} 10 TCD$_{50}$/ml of cell culture fluid medium were obtained The cytopathic

TABLF 4

Diagnosis of Respiratory Disease Observed During the Spring of 1962

Diagnosis	Total number of cases (54)	Number of cases associated with ECHO 28 infection (10)
Minor respiratory illness	34	8
Pharyngo-conjunctivitis	9	1
Exudative pharyngitis	3	0
Acute respiratory disease	*8*	*1*

DISCUSSION

The picorna virus group comprises enteroviruses and recently the rhino (11) and coryza viruses (12) The classification is based on physico-chemical properties of the virus (13) The clinical entities associated with infection with picorna-virus present a more heterogeneous picture Some types within the enterovirus group have been shown to cause aseptic meningitis The enteroviruses associated with respiratory disease, especially Coxsackie A21 and ECHO 28, show clear differences in basic properties Coxsackie A21 is acid stable (12), inhibited by 2α hydroxybenzylbenzimidazol (HBB) and guanidine (14), but ECHO 28 shows the reversed character of these three properties (12, 14) Thus a classification only based on association with type of clinical disease appears unsatisfactory

An outbreak of minor respiratory disease in a military camp in Uppsala Sweden, during the spring of 1962 has been shown to be associated with an infection of ECHO 28 virus The number of virus isolates was only 2 of 10 cases with significant antibody rises The use of confluent monolayers with only marginal cytopathic effects of the virus and the incubation at 37° C at primary isolation of the virus may have accounted for the low isolation frequency Since the affected conscripts only showed significant antibody rises to ECHO 28 virus, although the antibody response to a number of viral and bacterial antigens was tested, an association between the FCHO 28 infection and the disease appears likely

SUMMARY

The association between an infection of ECHO 28 virus and an endemic outbreak of minor respiratory disease in military recruits is described

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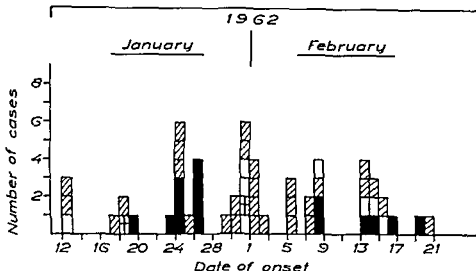


Fig 1

Incidence of respiratory disease at different dates during the period January 12–February 15 1962

- Cases with fourfold increase in neutralizing antibodies against ECHO 28
- Cases with antibody levels above 1/64 against ECHO 28 in all three serum samples
- Cases with low antibody levels against ECHO 28 in all three serum samples
- Denotes virus isolations of ECHO 28

observed were nasal discharge, injection of the pharynx and in single cases fever above 38° C When the cases were distributed according to different categories of respiratory disease, most of the conditions observed were classified as minor respiratory illness, as seen in Table 4 The classification used is based upon the differentiation earlier described (8) and originally reported by *Grieble et al* (10)

TABLE 3

Bacteriological Findings and Significant Antibody Response to Bacterial Antigens in the Recruits with Respiratory Disease

Bacterial type	Total number of cases (51)	Number of cases associated with ECHO 28 infection (10)
Staph aureus	27	4
Haemolytic streptococci	13	2
Pneumococci	4	1
H influenzae	5	0
Meningococci	7	1
Type of antibody		
Antistaphylolysin	0	0
Antistreptolysin	1	0
Antipneumolysin	0	0
H influenzae CF antibodies	0	0

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FRACTIONATION OF STAPHYLOCOCCAL TOXINS BY GEL-FILTRATION

By

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Received 31 v 63

Little is known about the part played by the extracellular products of *Staphylococcus aureus* in inducing illness in the host organism One exception is the enterotoxin produced by certain strains, which may cause acute food poisoning and, in patients treated with antibiotics, enterocolitis (*Elek* 1959, *Kienitz* 1962) In order to determine the attack mechanisms and the significance of other toxins it is important to obtain these substances in their pure state In the case of enterotoxin the preparation of a pure form would also aim at the establishment of a simple method for the diagnosis of enterotoxin-producing strains, at present dependent on experiments with cats and monkeys There are several ways of purifying different extracellular products of *S aureus*, some yielding immunologically pure products others not (*Elek* 1959) However, in many cases these procedures entail great losses in activity or are unsuitable for preparation on a large scale The present essay describes the fraction ition of staphylococc il toxins on crosslinked dextrans

MATERIAL AND METHODS

Stra n Th S c . *Evans* 1947 was
us 53)
(*Casman* 1958)
en al concentration
of 1 per cent calculated on the dry weight of the dialysate The proteospeptone was dialysed in vacuum according to *v Hofsten et al* (1960) After dialysis the dialysate percolated through a Seitz filter

The culture itself was made in three stages At stage I 100 ml retorts containing sterile glassbeads were used During incubation these were kept in a thermostat resting on a slant
of a colony
formed the
CO_2 accord
Stockholm
in a slight
t tal volum and the supernate evaporated at +9° C.

Preparation of Crude Toxin for Fractionation

100 g of the dry supernate was dissolved in 200 ml of aq dest and dialysed against 0 02 M Na PB Concentration was carried out by evaporation in a flash evaporator

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Leucocidin

Leucocidin was determined according to a method described by *Gladstone et al* (1957) One drop of blood from a human fingertip was permitted to clot on a cover glass which was placed in a moist chamber (37° C) for 30 minutes The clot and erythrocytes were washed away with buffered saline containing 0 5 per cent gelatin The granulocytes adhere to glass and therefore stayed behind on the cover glass One drop of the sample was put on a slide and covered with the cover glass bearing the granulocytes In order to prevent evaporation the edges of the cover glass were sealed with paraffin The sample was then incubated for 20 minutes at 37° C If leucocidin (Panton Valentine) is present morphological changes take place that can be distinguished e g from those of leucolysin (= delta haemolysin) The changes were observed in a phase contrast microscope

Lipase

Lipase plates with basins were poured using matrices for basin plate technique according to Ouchterlony The agar (2 per cent) contained 1 per cent Tween 20 and 0 01 per cent $CaCl_2$ (*Jessen et al* 1957) The samples were allowed to drip into the basins and the plates left to stand at 37° C. In the presence of a diffusable lipolytic enzyme crystals of insoluble fatty acid salts were formed around the basins The lipase titre indicates the maximum dilution of the sample at which a dense ring 1 mm broad is still formed around the rim of the basin after 6 hours

Phosphatase

The quantity of alkaline phosphatase was determined with p nitrophenylphosphate as a substrate at pH 10 5 The amount of liberated p nitrophenyl was measured spectrophotometrically at 400 mμ 3 ml 0 02 N NaOH having been added to the column eluate For determination of the number of sigma units in the crude toxin 10 ml 0 02 N NaOH was added (Sigma Chemical Company)

Activity Recovery

Activity recovery was calculated according to titrations of the primary material as well as the eluate after the active fractions had been added together

Gel Filtration

Columns of different sizes were tested They were packed and prepared according to *Flodin* (1962) According to the situation Sephadex types (AB Pharmacia Uppsala 1959 1962) G–50 G 75 G 100 or G 200 with a water regain of 5 0, 7 5 10 and 20 resp [illegible]
Th [illegible]
dif [illegible]

a Beckman DU spectrophotometer

TABLE 1

	S-6-strain	Supernate
Coagulase	+	—
enterotoxin gel diffusion	+	+
cat test	+	+
fibrinolysin	+	+
alpha haemolysin	+	+
beta haemolysin	—	—
delta haemolysin	+	+
hyaluronidase	+	+
leucocidin	+	+
alkaline phosphatase	+	+

at +30° C Vacuum dialysis under refrigeration proved unsatisfactory since the dialysis tubing let out enzymes in such quantities that they could be detected immunologically in the eluate Compared to selective precipitation methods this procedure involved small losses in activity The concentrate was dialysed to correspond to whatever buffer was to be used in the experiment in question

Determination of Toxin and Enzyme Activities

Coagulase

The formation of coagulase was determined by incubating 0 1 ml of broth culture (18 hours) for 1 hour in 0 9 ml Difco coagulase plasma at 37° C Free coagulase was determined by incubating equal parts of millipore filtered samples and Difco *coagulase plasma for 1 hour at 37° C* The formation of a clot indicated coagulase (*Duthie* 1954)

Enterotoxin

1) *Test in vitro* The frac
double diffusion technique
being used The fractions w
courtesy of Dr M S Bergdoll Chicago

2) *Biological test* The cat test according to *Hammon* (1941) was used The sample *was boiled for 30 minutes centrifuged and injected into the femoral vein of a cat,* 2 hours after the animal had been fed If the cat vomited within 2 hours the test was considered positive As a rule the cats had been at the Institute at least 1 month before the tests No more than maximum 3 toxin tests were carried out on each individual

Fibrinolysin (Staphylokinase)

The substrate for the fibrinolysin plates was obtained by mixing without shaking 85 parts 56° heart infusion agar with 15 parts sterile rabbit plasma (*Christie et al* 1941) The samples were allowed to drip into the basins and incubated at 37° C In the presence of fibrinolysin a clear zone appeared around the rim of the basins The fibrinolysin titre indicates the maximum dilution of the sample at which a clear zone 1 mm broad is still formed after 6 hours

Haemolysins

1) The alpha haemolysin titre was measured by incubating 0 5 ml of a 1 per cent suspension of washed rabbit erythrocytes with 0 5 ml of serial dilutions of the sample to be investigated The titrations were read after 1 hour at 37° C and 1 hour at room temperature The highest toxin dilution still allowing complete haemolysis was taken to be the titre (*Kumar et al* 1962)

2) The beta haemolysin titre was determined as above with two modifications Sheep erythrocytes were used instead of rabbit ones and during the second hour the samples stood in a refrigerator at +4° C instead of room temperature (*Kumar et al* 1962)

3) *Delta haemolysin was measured in the same way as alpha* haemolysin but human erythrocytes type O Rh+ were used instead of rabbit ones (*Kumar et al* 1962)

Hyaluronidase

Hyaluronidase was determined by a so called mucin clot prevention test Hyalu
' complex from forming a typical mucin
ample was added 1 ml of hyaluronic acid
olution hyaluronic acid in aq dest + 10
+ 20 parts aq dest The preparation was
incubated for 20 minutes at 37° C After cooling in ice 1 drop of acetic acid was added and the result registered immediately By the hyaluronidase titre is meant the highest *dilution* of the sample still preventing the formation of a mucin clot (*McLean et al* 1943)

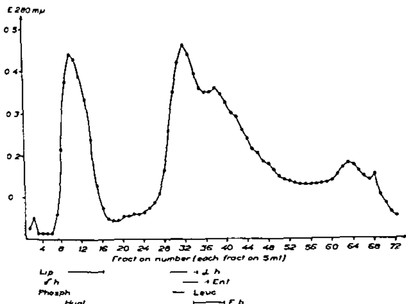


Fig 2

Fractionation of crude toxin on Sephadex G 100 in 0.05 M NaPB with 0.5 M NaCl pH 7.2. For key see Fig. 1

hyaluronidase alpha haemolysin leucocidin enterotoxin fibrinolysin. No activity was found after fraction 48 Fig. 1. The absorption peak in fractions 48-50 may therefore represent substrate remnants not removed by dialysis.

A better separation especially of the second peak (fractions 38-46) Fig. 1 was obtained by raising the molarity of the buffer. Fig. 2 shows a filtration through Sephadex G 100 in 0.05 M NaPB with 0.5 M NaCl pH 7.2. In this way alpha haemolysin and leucocidin on the one hand were completely separated from fibrinolysin on the other. Between these two activities but with overlapping in both directions lay enterotoxin. However the improved separation with high salt concentrations was combined with losses in activity especially concerning delta haemolysin and phosphatase. Both were tested after dialysis with buffered saline.

In order to make separation still more effective Sephadex gels of different porosities were tried. The first peak from G 100 representing high molecular substances (fractions 21-26 Fig. 1) was concentrated, dialysed and filtered through Sephadex G 200 in a 30 × 2 cm column in 0.02 M NaPB pH 7.2 (Fig. 3). This type of Sephadex excludes substances with molecular weights exceeding roughly 200 000. Delta haemolysin lipase and phosphatase all passed out in connection with the void volume from which it may be concluded that their molecular weights are more than roughly 200 000.

The second peak from G 100 (fractions 38-46 Plate 1) was filtered

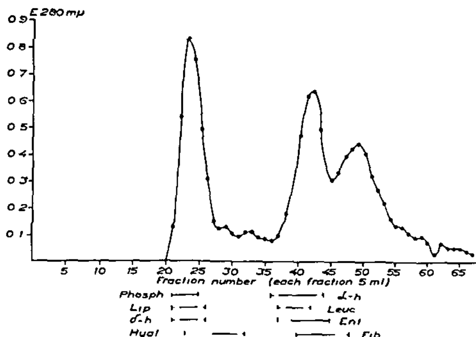


Fig 1

Fractionation of crude toxin with Sephadex G-100 in 0.02 M NaPB pH 7.2

Phosph	= Alkaline phosphatase
Lip	= lipase
δ-h	= delta haemolysin
Hyal	= hyaluronidase
α h	= alpha haemolysin
leuc	= leucocidin
ent	= enterotoxin
fib	= fibrinolysin
	= traces of activity
———	= ca 90 per cent of the activity

RESULTS

The bacteria as well as their extracellular environment were tested for a number of enzymes and toxins associated with *S aureus* The results appear in Table I Coagulase occurred in the bacteria suspension only, while the other enzymes and toxins were found in both tests

To begin with concentrated dialysed crude toxin corresponding to ca 1 l cultures was filtered through Sephadex G-100 in 0.02 M NaPB pH 7.2 The column measured 50 × 3.5 cm The activities were distributed over the different fractions as shown by Fig 1

The first peak (fractions 21–26, Fig 1) comprising delta-haemolysin, lipase and phosphatase, occurred immediately after the void volume of the column and represents substances with molecules too large to pass into the network of the gel For Sephadex G 100 this means substances with a molecular weight of about 100 000 or more Thereupon followed activities representing substances that had entered the meshwork of the gel Their molecular weights are less than ca 100 000 and they pass into the eluate in order of size, the largest molecules first

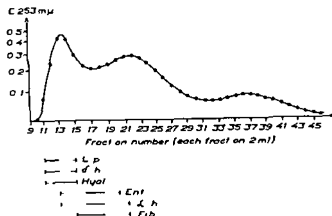


Fig 5

Fracti nation of crude toxin on Sephadex G 75 in 0 02 M NaPB pH 7 2
For key see Fig 1

through Sephadex G 50 under the same conditions as above Alpha-hae molysin leucocidin, enterotoxin and fibrinolysin all passed out with the void volume (Fig 4) and their molecular weights can therefore be roughly said to exceed 8000 10 000

TABLE 2

Molecular Weight		
10000 ,0000	~ ,0000	> 200000
fibrinolysin enterotoxin leucocidin alpha haemolysin	hyaluronidase	delta haemolysin lipase alkaline phosphatase

TABLE 3

	Primary titre	Recovery
fibrinolysin	1/456	50 %—(100 %)
alpha haemolysin	1/4560	100 %
delta haemolysin	1/100	100 %
hyaluronidase	1 640	50 %—(100 %)
lipase	1/320	100 %
alkaline phosphatase	6 sigma units	10 %

Fig 5 shows a fractionation of crude toxin with Sephadex G 75, the conditions being the same as described for G-100 Alpha haemolysin, enterterotoxin, and fibrinolysin all passed into the meshwork which seems to show that their molecular weights are lower than ca 40 000 50 000 The activity maxima for all three substances lay in the same frac tion In this case G 75 was clearly not as suitable for separation as Se-

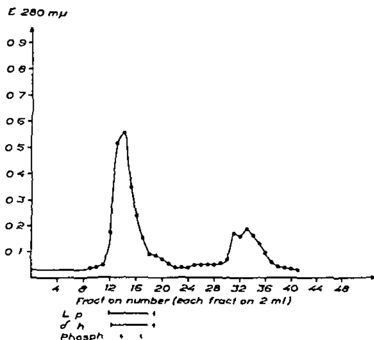


Fig 3

Fractionation of fractions 21 26 (Fig 1) on Sephadex G 200 in 0 02 M NaPB pH 7 2
For key see Fig 1

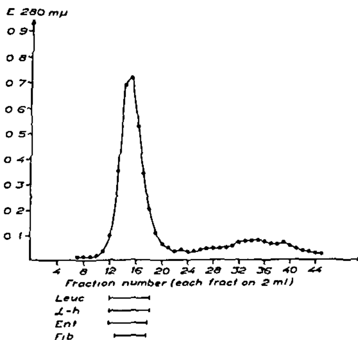


Fig 4

Fractionation of fractions 38–46 (Fig 1) on Sephadex G 50 in 0 02 M NaPB pH 7 2
For key see Fig 1

quired, by means of which particles with molecular weights in the region above 200 000 can without damage be separated from one another In preliminary experiments with pearlcondensed agar it has been possible to separate delta-haemolysin and lipase (*Hallander &Bengtsson*, unpublished data)

The procedure described above gave no immunologically pure fractions only a rough division according to molecular size However the method has offered a complete or almost complete recovery for most of the activities The exception is alkaline phosphatase The procedure was devised as a first step in a purifying technique for enterotoxin It is, however, equally suitable as a gentle initiation in preparing other extracellular products in their pure form

SUMMARY

By means of gel-filtration on cross-linked dextrans (Sephadexes) staphylococcal toxins have been fractionated into three main groups 1) delta haemolysin, lipase alkaline phosphatase 2) hyaluronidase 3) alpha-haemolysin, leucocidin, enterotoxin and fibrinolysin (staphylokinase) At high buffer molarity alpha-haemolysin and leucocidin on one hand have been perfectly separated from fibrinolysin on the other Enterotoxin has been found between these and overlapping to either side A rough estimate of the molecular weights has been made

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phadex G-100. Hyaluronidase was found in the void volume or immediately after together with lipase, and its molecular weight is therefore around 50 000

In Table 2 the various substances are grouped according to their approximate *molecular weights*, based on the rough estimates possible with the use of Sephadex gels (Pharmacia AB 1959, 1962).

The Sephadex gels turned out to do very little damage No regular measurements of activity recovery were made Table 3 shows the *activity recovery* after a fractionation with Sephadex G-100 in 0 02 M NaPB with 0 1 M NaCl pH 7 2 For hyaluronidase and fibrinolysin 50 per cent recovery was the minimum value, since the difference between 50 and 100 per cent represented only one tube in the serial dilution For alkaline phosphatase however the activity recovery was only 10 per cent

DISCUSSION

"The sephadexes consist of cross-linked dextran chains with random ether linkages to the glucose residues in the polysaccharide chains — By varying the degree of cross-linkages in the manufacturing process, different porosities of the network are obtained" (Pharmacia AB 1962)

With Sephadex G-100 a group of high molecular substances representing delta-haemolysin, lipase and phosphatase were separated from a group of smaller substances that passed into the network of the gel These latter were fairly well separated from one another also in their own group Thus hyaluronidase stood alone in the shadow of the first high molecular peak but with its maximum separate from the latter Of the others alpha-haemolysin and leucocidin on the one hand were quite separate from the smaller fibrinolysin on the other This separation was obtained by raising the salt content Between the two groups and overlapping to either side there was enterotoxin

Sephadex G-100 is obviously the sephadex type which irrespective of molecular range offers the best conditions for separating the extracellular products of *staphylococcus aureus*

Since separation proceeds according to molecular size a good picture was also obtained of how the various substances were arranged in this respect For a rough estimate of the absolute molecular weight of the fractions the other sephadex types were a useful complement

The upper limit of sephadex capacity lies around *M* 200 000 Substances with a higher molecular weight pass out in the void volume of Sephadex G-200 It is therefore natural that delta-haemolysin, lipase and phosphatase had a common activity maximum However the fact that in *Clostridium perfringens A* the alpha haemolysin is actually a lecithinase C (*MacFarlane et al* 1941) gives rise to the interesting speculation that *e g* the lipase and delta-haemolysin activities may be represented by a single enzyme In order to investigate this, methods are re-

BRIEF REPORTS

BACTERIAL CONTAMINATION AS THE CAUSE OF A HIGH CONCENTRATION OF SPECIFIC BLOOD GROUP SUBSTANCE IN THE PLASMA

By *Hans Hostrup*

In most of these cases, the samples originated from patients with disorders which per se contributed to the explanation of the phenomenon or were cord blood in which the increased concentration was presumably due to admixture of amniotic fluid However two of the cases on record occurred in samples from healthy donors (*Salmon & Malassenet* 1960 *Hostrup* 1963) In these two cases the explanation may possibly be sought in admixture of substance containing material

In order to demonstrate how this may occur some experiments were performed in which group B blood was contaminated with a bacterial culture containing group B substance In the study of a large number of various Gram negative bacteria *Springer et al* (1961) demonstrated A B and H substances in high concentrations in a number of strains

In the present experiments a culture of E coli O_{86} which was kindly supplied by *G F Springer* was used Eleven successive subcultures were made on a synthetic medium which did not contain B substance In an eight hour culture containing about 845 × 10 bacteria per ml inhibition technique revealed a strong specific inhibition of anti B After removal of the bacteria by vigorous centrifugation the medium in which the bacteria had been grown showed unchanged inhibition

Ten samples of blood from each of three group B donors stabilised by means of ACD (Ph Dan) were used in the experiments The samples from each donor were divided into two groups each of five tubes which were inoculated with 20 000 10 000 5 000 1 000 and O bacteria per ml respectively One group was stored at 4° C, the

had been frozen since the blood taking The erythrocytes were studied against anti A anti B and in AB serum and the results were compared with those of a similar study of fresh erythrocytes from the donors

Results After eight days, bacterial growth was revealed in all the contaminated tubes of the group which had been stored at 4° C The viable bacterial count had decreased The plasma content of B substance was unchanged and the antigenic properties of the erythrocytes remained unaffected by the contamination

In the group which had been stored at room temperature bacterial growth was observed in the samples from two donors but not in those from the third In tubes with more than 100×10^6 bacteria per ml the plasma content of B substance showed a definite increase and the most heavily contaminated tubes also revealed haemolysis and clotting The increase in the plasma content of B substance could also be recognised in the less heavily contaminated tubes without haemolysis and clotting In the blood samples from the third donor which showed no bacterial growth the plasma content of B substance remained unchanged

The B antigen of the erythrocytes was weakened in the tubes which had been

Received 10 vii 63 *from the Blood Bank and Blood Grouping Laboratory (Chief F Kissmeyer Nielsen M D) Aarhus Kommunehospital and the Department of Bacteriology (Chief Professor A Stenderup M D) the Institute of General Pathology University of Aarhus Aarhus Denmark*

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S	s	SS	Ss	ss
31.38	68 62	9 85	43 06	47 09

The separate *M* and *N* gene frequencies obtained in this investigation (*M* 54 96 %, *N* 45 04 %) are in agreement with those previously found in Sweden (*M* 56 99 %, *N* 43 01 %) by *Beckman* (1959)

On the basis of 193 persons tested *Allison et al* (1956) calculated the following chromosome frequencies in Swedish Lapps

MS	*Ms*	*NS*	*Ns*
25 06 %	13 54 %	19 65 %	41 75 %

As could be expected from investigations concerning other blood group systems, these frequencies differ considerably from those stated in the present paper concerning the remaining part of the Swedish population

TABLE 1

The MNSs Genotype Frequencies in the Swedish Population

	Genotypes %	Phenotypes absolute		χ^2
		Exp	Obs	
MS/MS	4 80	122 43	124	0 020
MS Ms	14 48			
Ms Ms	10 92	69.34	74	0 313
MS/NS	4 15			
MS Ns	15 59	165 10	164	0 007
Ms/NS	6 26			
Ms Ns	23 51	149 29	138	0 854
NS/NS	0 90	48 51	48	0 005
NS Ns	6 74			
Ns Ns	12 65	80 33	87	0 554
	100 00	635 00	635	1 753

$\chi^2 = 1\,753 \quad d\,f = 2 \quad 0\,5 > P > 0\,3$

References 1 *Allison A C Broman B Mourant A E & Ryttinger L* J Roy Anthr Inst *86* 87 1956 —2 *Beckman L* Hereditas 45 1 1959 —3 *Boyd W C* Science *118* 756 1953 4 *Boyd W C* Amer J hum Genet *6* 1, 1954 —5 *Boyd W C* Acta Genet Med Gemell *5* 234 1956 6 *De Groot M H* Amer J hum Genet *8* 229 1956 —7 *De Groot M H & Li C C* Ann Hum Genet 24 109 1960 — 8 *Landsteiner K & Levine P* J exp Med 47 757 1928 —9 *Levine P Kuhmichel A B Wigod M & Koch E* Proc Soc exp Biol NY 78 218 1951 —10 *Mourant A E* The Distribution of the Human Blood Groups Blackwell Scientific Publications Oxford 1954 11 *Race R R & Sanger R* Blood Groups in Man 4th ed Blackwell Scientific Publications Oxford 1962 —12 *Sanger R & Race R R* Amer J hum Genet 3 332 1951 13 *Walsh R J & Montgomery C* Nature *160* 504 1947 14 *Wiener A S* Acta Genet Med Gemell 3 314 1954 15 *Wiener A S Unger L J & Gordon E B* J Amer Med Ass *153* 1444 1953

stored at room temperature, independent of the contamination. The erythrocytes had not become polyagglutinable.

Conclusions. Contamination of group B blod samples with E. coli O_{86} which contains B substance, may lead to an increase in the plasma content of B substance.

This observation shows that in the handling of blood samples, strict sterile precautions are of the greatest importance and that the time during which the samples are allowed to remain outside the refrigerator should be reduced to a minimum.

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MNS BLOOD GROUP FREQUENCIES IN THE SWEDISH POPULATION

By *Aage Heiken*

According to the genetic interpretation of the MNSs blood group system put forward by Sanger & Race (1951) there exists a close linkage between the two autosomal pair of alleles *M, N* (*Landsteiner & Levine* 1928) and *S, s* (*Walsh & Montgomery* 1947, *Levine et al.* 1951) resulting in the occurrence of the following four chromosomes (gene complexes): *MS, Ms, NS* and *Ns*. The frequency of these chromosomes has been calculated in many populations on the basis of test with anti-M, anti-N and anti-S sera. As stressed by *Race & Sanger* (1962) this may be done provided the population is European. The occurrence of the U antigen (*Wiener et al.* 1953) will prevent such a simplification in populations of African origin.

Since MNSs frequencies (with the exception of Lapps) so far have not been published in the Swedish population, it was found of interest to give the data obtained *at the State Institute for Blood Group Serology in connection with paternity cases.*

During the last six months blood samples from 635 unrelated adult persons from all parts of the country have been investigated with anti-M, anti-N and Anti-S sera. The erythrocytes were tested for the presence of the S antigen with an iso-immune test serum. The saline test tube method with centrifugation (1000 RPM) was used. If no reactions were obtained, the cells were incubated for 30 minutes at 30° C and centrifugation was repeated. The M typing was made on slides with two different rabbit immune test sera and so was the N typing. The M and N results were checked by absorption tests. All test sera used in the investigation were checked by known test cells.

The following phenotype frequencies were found:

MS+	MNS+	NS+	MS	MNS	NS
19.53 %	25.83 %	7.56 %	11.65 %	21.73 %	13.70 %

On the basis of these figures the chromosome frequencies were calculated according to the method given by Mourant (1954; other methods of calculation of frequencies have been worked out by *Boyd* 1953, 1954, 1956, *Wiener* 1954, *De Groot* 1956, *De Groot & Li* 1960).

MS	Ms	NS	Ns
21.91 %	33.05 %	9.47 %	35.57 %

The genotype frequencies are presented in Table 1.

If the analysis is confined to S and s, the following gene and genotype frequencies can be calculated:

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quency was seen in the biopsy taken 72 hours after irradiation About 10 per cent of the mitoses in the two first mentioned categories were found to present one or more aberrations

The types of chromosome aberrations demonstrated could be classified into a few main groups Translocation was manifest in the form of chromosomes in which one pair of arms was appreciably longer than that of the homologous chromosome Reliable registration was possible only of translocation to the chromosome numbers 1 2 4–5 and 13–15 (Denver classification) A series of mitoses was found con

time being these variations have not been classified as aberrations Some aberrations in the form of pericentric inversions were visible but only on the large chromosomes Deletion represented a common type of aberration, generally the site of fragmentation on one pair of arms would be localized close to the centromere

less important aberrations were observed but the interpretation of this finding is not possible until a more comprehensive material is available—It should be noted in particular that neither ring development nor dicentrics were observed

Thus *in vivo* irradiation (100 r) of skin is found to induce the development of a number of chromosome aberrations persisting in cell cultures after cultivation for long periods (2 4 weeks)

References 1 *Bender M A & Gooch P C* Radiat Res *16* 44–53 1962—2 *Bender M A & Gooch P C* Radiat Res *18* 389 396 1963—3 *Buckton K F Jacobs P A Court Brown W M & Doll R* Lancet *ii* 676–682, 1962—4 *Moore J G van Campenhout J L & Brandkamp W W* In press—5 *Tough I M Buckton K F Baikie A G & Court Brown W M* Lancet *ii* 849 851 1960

PERSISTING CHROMOSOME ABERRATIONS IN CELL CULTURES FROM IRRADIATED HUMAN SKIN

By *Jakob Visfeldt*

Previous studies on radiation induced chromosome aberrations have been based mainly on irradiation of established cell cultures followed by scoring during the next cell division at which stage it is possible to register all of the induced aberrations If scoring were carried out during later cell divisions unknown numbers of aberrations might be lost because many damaged cells are destroyed after one or a few, mitoses

Hence essential importance must be attached to the solution of the question which radiation induced aberrations may be present without destroying the capacity of cells to reproduce in human tissue and cultures? In special circumstances assumedly these cells may become of a pathogenic nature in man either in his own or in the following generation provoking malignant conditions or congenital malformations respectively

So far few reports only are available discussing the matter of persisting chromosome aberrations in man following irradiation *Tough et al* (5) and *Buckton et al* (3) have described aberrations in leucocytes from peripheral blood of patients exposed to irradiation of the spine on account of ankylosing spondylitis *Bender & Gooch* (1 2) have examined eight males who received Whole body irradiation in a critical accident Chromosome aberrations were found to persist in the peripheral leucocytes 29 and 42 months after irradiation *Moore et al* (4) describe persisting aberrations in the leucocytes in patients exposed to radiation treatment on account of malignant gynaecological disorders—The here quoted authors have recorded primarily mitoses presenting deviating chromosome numbers, rings dicentrics and fragments Also a few other aberrations known from radiation experiments on drosophila and tradescantia have been observed

At present experiments have been initiated in our laboratories the object of which is to examine the persisting chromosome aberrations in human skin exposed to radiation The first experiment was carried out on a patient who on the diagnosis of laryngeal cancer was due to heavy therapeutical x ray irradiation Prior to the institution of treatment the patient received 100 r by 100 kv on a 5 cm field on the right forearm In addition to the control biopsy taken from the left arm biopsies were taken at intervals of 3 24 and hours from the irradiated field The skin biopsies were cultured and the rate of growth registered The biopsy taken 24 hours after exposure was found to grow appreciably better than any of the other biopsies 733 cells from the four cultures were analysed in late metaphase Mitoses of high quality were used exclusively If it could not be finally decided whether or not an aberration was genuine it has been preferred in all cases to classify the mitoses as normal All cells have been analysed personally by the author and with the exception of cells presenting chromosome numbers other than 46 all of the aberrations observed have been submitted to the inspection of one or several colleagues engaged in work with chromosome analyses There is a concensus of opinion as to the correctness of interpretation in all cases

Results Minor numbers of chromatid aberrations were noted both in the irradiated biopsies and in the non exposed one Any difference whether qualitative or quantitative could not be definitely ascertained Various different chromosome aberrations were demonstrable in the irradiated biopsies but none in the non exposed specimen The frequency of mitoses involving aberrations was highest in slides from the biopsy taken 24 hours after irradiation it was slightly lower in those from the biopsy taken 3 hours after irradiation a considerably lower fre

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Received 21 xi 63 from *The University Institute For Human Genetics Department Of Experimental Genetics And Cytology (J Schult Larsen M D) and from The Radium Centre Copenhagen (Professor Jens Nielsen M D)*

This work has been supported by grants from the Danish Atomic Energy Commission and the Danish Anti Cancer League

cortex and is demonstrable also in the reticulo endothelial cells of the marrow The substance in the cortex increases in concentration with the age of the animals Treatment with ACTH produces a marked increase of the substance in the fascicular zone but only a slight change in the glomerular zone Hydrocortisone treatment gives a pronounced aggregation of the granules in all the layers

G F Voigt EXPERIMENTAL MANGANESE SULPHATE POISONING IN GOLDEN HAMSTERS

Golden hamsters are much more sensitive to repeated subcutaneous injections of manganese sulphate than mice rats or guinea pigs After injection of 4 5 mg manganese sulphate /100 g bodyweight daily for 6 days the hamsters on the seventh day showed severe necrosis of the acinoperipheral liver cells with accumulations of polymorphonuclear leukocytes, necrosis of epithelial cells in Henles' loop, distal convoluted tubules and collecting tubules of the kidneys, perivascular infiltration of leukocytes in the pancreas and small necroses in the myocardium

Simultaneous injection of zinc sulphate, 4 5 mg/100 g bodyweight prevented fairly effective the liver damage otherwise induced by the manganese sulphate There is probably competitive inhibition between manganese and zinc

C G Ahlström H Grundsell & G Sköld ROUS SARCOMA IN HYDROCORTISONE-TREATED RATS

New born rats were given 2 5 mg hydrocortisone subcutaneously and at the same time inoculated with finely minced Rous chicken sarcoma suspended in Hank's solution A second dose of hydrocortisone was given one week later All of the hydrocortisone treated rats developed tumours at the site of inoculation against only five out of thirteen controls Moreover the period of latency was shorter and almost all rats developed multiple tumours whereas the controls only showed solitary tumours 40 days after the inoculation

In the hydrocortisone treated rats the tumour nodules first appeared around remnants of injected chicken tumour material Chromosome analysis of the tumours showed chromosomes of rat type In gel precipitation tests no antigen common to the rat tumours and Rous chicken sarcoma could be demonstrated

No difference in the percentage of takes or in the period of latency was seen in cortisone treated new born rats injected with a cellular suspension or with an aliquot of homogenized material from the Rous chicken sarcoma

It seems possible that the effect of cortisone is related not only to its power to inhibit antibody formation but also to its antiphlogistic effect which may permit the injected material to persist for a longer time than in the controls

F Brehmer Ar Iersson VASCULITIS ALLERGICA CUTIS

The name Vasculitis allergica [illegible] pur-pura rheumatica anaphylactoid [illegible] ourge rot's syndrome Clinically five [illegible] papules petechiae [illegible] dull vesicles and necrosis These can occur independently or together in different combinations Simultaneously with the cutaneous changes SR increase electrophoretic changes haematuria or symptoms of joint disease can occur Cutaneous vasculitis is common to all clinical variants Usually small vessels in the corium are affected but sometimes also larger subcutaneous vessels are involved Typical changes consist of infiltrations of polymorphonuclear leukocytes in

TRANSACTIONS
OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting April 20, 1963

B. Thorell: THE DISTRIBUTION OF DIFFERENT TYPES OF HEMOGLOBIN WITHIN THE ERYTHROCYTES

G. Nathorst-Windahl: STUDIES ON PANCREATECTOMIZED DIABETIC RABBITS

Diabetes in pancreatectomized rabbits has been studied in 71 animals. The pancreatectomy has to be done in two stages in order to avoid a very high mortality rate.

Subtotal pancreatectomy results in a mild type of diabetes: the animals can live for extended periods without insulin administration and they do not lose weight. During fasting blood sugar falls to normal level and ketonuria does not appear. When food is given hyperglycemia and glycosuria reappear.

Total or approximately total pancreatectomy gives a severe diabetes with great loss of weight, hyperlipemia and marked ketonuria. Insulin administration is necessary to avoid death in acidosis and coma. Fasting in these rabbits gives a rise in blood sugar and as a rule hyperlipemia and ketoacidosis.

Digestion trials have been carried out with the animals on a normal diet. There is a slight but statistically significant decrease in the digestibility of protein in the pancreatectomized group but no changes in the digestibility of fat (estimated as total fat in an ether extract) or carbohydrate (crude fiber and N free extract).

G. Hansson & L. Angervall: THE PARATHYROID GLAND IN RATS WITH ALLOXAN DIABETES

C. Ahrén, O. Hansson & I. Kvernes: VARIATIONS IN VOLUME AND SHAPE OF THE NUCLEI IN ZONA FASCICULATA DURING DIFFERENT DEGREES OF ADRENAL ACTIVITY IN RATS

L. Angervall & P. Lundin: HORMONAL INFLUENCE ON FETAL RAT THYMUS

U. Brunk: SULPHIDE SILVER METHOD ON CRYOSTAT SECTIONS OF HAMSTER ADRENAL

Physical development (sulphidesilver method) of hydrogensulphid gas treated sections of hamster adrenals reveals two substances. One of these is probably copper and occurs in the marrow cells, in the centrally situated cells in a high concentration but in only insignificant amounts in the peripheral cells.

The other substance has not yet been identified but it might be a metal with a readily oxidized sulphide. Since it is insoluble in water it can not be ascorbic acid and it can only be demonstrated in cryostat cut but not in paraffin embedded material. The substance occurs in varying concentration in the different layers of the

cortex and is demonstrable also in the reticulo-endothelial cells of the marrow. The substance in the cortex increases in concentration with the age of the animals. Treatment with ACTH produces a marked increase of the substance in the fascicular zone but only a slight change in the glomerular zone. Hydrocortisone treatment gives a pronounced aggregation of the granules in all the layers.

G. E. Voigt: EXPERIMENTAL MANGANESE SULPHATE POISONING IN GOLDEN HAMSTERS.

Golden hamsters are much more sensitive to repeated subcutaneous injections of manganese sulphate than mice, rats or guinea pigs. After injection of 4.5 mg manganese sulphate /100 g bodyweight daily for 6 days the hamsters on the seventh day showed severe necrosis of the acinoperipheral liver cells with accumulations of polymorphonuclear leukocytes, necrosis of epithelial cells in Henles loop, distal convoluted tubules and collecting tubules of the kidneys, perivascular infiltration of leukocytes in the pancreas and small necroses in the myocardium.

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C. G. Ahlström, H. Grundsell & G. Sköld: ROUS SARCOMA IN HYDROCORTISONE TREATED RATS.

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It seems possible that the effect of cortisone is related not only to its power to inhibit antibody formation but also to its antiphlogistic effect which may permit the injected material to persist for a longer time than in the controls.

F. Brehmer Andersson: VASCULITIS ALLERGICA CUTIS.

The name Vasculitis allergica cutis includes different clinical concepts, e.g. purpura rheumatica, anaphylactoid purpura, periarteritis nodosa cutanea and Gougerot's syndrome. Clinically five types of cutaneous changes exist: maculopapules, petechiae, noduli, vesicles and necrosis. These can occur independently or together in different combinations. Simultaneously with the cutaneous changes SR increase, electrophoretic changes, haematuria or symptoms of joint disease can occur. Cutaneous vasculitis is common to all clinical variants. Usually small vessels in the corium are affected but sometimes also larger subcutaneous vessels are involved. Typical changes consist of infiltrations of polymorphonuclear leukocytes in

the vascular walls and surroundings and leukocytoclasis, bleeding and fibrinous exudation in the connective tissue Often some vessels show fibrinoid necrosis or thrombosis Sometimes vascular changes lead to epidermal infarction with blister formation or necrosis It is important to take fresh lesions for histological examination older lesions are often secondarily infected

Clinically and histopathologically, the picture resembles a collagen sis The condition must probably be considered an allergic reaction The prognosis regarding the cutaneous disease is good, however vasculitis allergica cutis is often found to be part of another and serious condition

Seven cases of vasculitis allergica cutis were reported

K F Hogeman & G Ostberg UNILATERAL MAMMARY HYPERPLASIAS

Three cases of unilateral mammary hyperplasias were described

A girl of 17 had observed an enlargement of the left breast since 1½ year At operation an encapsulated tumour was found It weighed 480 g and was macr and microscopically a fibroadenoma of juvenile type

The other two cases were similar women of 40 and 35 years respectively married with two children each After the last lactation period the left breast had increased in size by one rather sudden by the other trough years At operation rounded encapsulated tumours were removed they weighed 1400 and 840 g respectively The cut surface was homogenous with small cysts Histological examination revealed a fibrous mammary tissue with widely spread partially normal partially atrophic glandular acini Widened ducts and cysts had a low atrophic epithelium

The later tumours show an unusual histological picture and are exceptionally large however they should be called fibroadenomas Similar cases seem not to have been reported earlier

J G Nordén ATYPICAL GLANDS IN THE COLON

Colectomy and ileorectal anastomosis was performed on a girl 18 years with familial adenomatosis and the colon showed like other such cases all transitions between single glands with atypical basophilic epithelium and fullgrown polyps At rectoscopy the rectum was likewise studded with polyps but 40 days later no polyp was to be seen Biopsies from normal looking mucosa contained several non polypous nests of atypical glands New biopsies 1½ months later showed only slight basophilia but no such atypical glands as earlier

The disappearance of polyps after colectomy has been reported earlier The pathogenesis of adenomas and carcinomas of the colon was discussed on the basis of the above facts and especially the possibility of endogenous initiation without morphological changes combined with an exogenous promotion (a cocarcinogenic factor in the colonic content)

S Hellsten MUCOCELE AND MUCINOUS ADENO CARCINOMA OF THE APPENDIX

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SUPPLEMENTA